FARE2012 WINNERS
Sorted By Study Section

NCI-CCR
CHEN, YUHONG
Postdoctoral Fellow
Biochemistry - General and Lipids
Fully synthetic virus-like nanoparticles targeting prostate cancer cells
Lack of selective delivery of therapeutic agents into the right organ and cells remains a major problem in therapy of many diseases, including cancer. Useful lessons in delivery can be learned from nature. Viruses are able to deliver a variety of biomolecules into certain cell types because they have the abilities to self-assemble and disassemble when needed and enter cells in receptor-mediated manner. However, assembly of molecules generated by chemical synthesis into virus-like particles has yet to be achieved. We have found that analogs of transmembrane (TM) helixes of integral membrane proteins self-assemble into round nanoparticles if equipped with terminal negative charges. A 24-amino acid peptide corresponding to the second transmembrane helix of the CXCR4 chemokine receptor adopts a predominantly beta-type conformation in aqueous solutions and self-assembles into nanoparticles with a diameter around 10 nm. Similar to viruses, nanoparticles fuse with cell membranes. Spontaneous fusion is accompanied by transition into active helical conformation that allows for potent inhibition of CXCR4 activity both in vitro and in a mouse model of metastatic breast cancer. Derivatization of CXCR4 TM antagonist with polyethylene glycol (PEG) chains slows down cell fusion, but results in nanoparticles that are very stable and more than 99% homogeneous in size. To generate nanoparticles that fuse with tumor cells in receptor-mediated manner, we constructed conjugates consisting of self-assembling CXCR4 antagonist, PEG chains and ligands for receptors overexpressed on prostate tumor cells, gastrin releasing peptide (GRP) receptor and luteinizing hormone-releasing hormone (LHRH) receptor. Attachment of peptide receptor ligands to the C-terminal end of CXCR4 TM antagonist not only did not interfere with self- assembly but produced more homogeneous and stable nanoparticles. Microscopy studies showed that targeted nanoparticles fused with receptor-positive cells much more effectively than with receptor-negative cells. In addition, nanoparticles have hydrophobic interior and can efficiently encapsulate hydrophobic anti-cancer agents. The new self-assembling virus-like particles present a new paradigm in drug development: a fully synthetic self-assembling delivery system with intrinsic dual and even triple anti-tumor activity. The approach is likely to be expandable to many tumor types and pathological conditions.

NICHD
Clokie, Samuel
Visiting Fellow
Biochemistry - General and Lipids
Discovery of a novel small RNA: piYRNA
The field of small RNAs has undergone rapid and intense study following the discovery of the mechanism of small RNA mediated gene silencing (siRNA) in the late 1990s. The subsequent discovery of a similar class of RNA termed microRNAs (miRNAs) has expanded the known complexity of RNA interference gene regulation still further. Another class of small RNAs revealed by recent studies is piRNA; these are ~28nt, have a different biogenesis compared to miRNA and are also involved in gene silencing. Here we report the discovery of several previously unreported small RNAs in the pineal gland, based on cloning and next-generation sequencing methodology. One of these, a 27nt RNA was selected for further study because of relatively high expression in comparison to other 'novel' small RNAs.

Alignment analysis indicated it maps to the stem-loop structure of the 112nt non-coding cytoplasmic RNA YRNA, consistent with a precursor/product relationship. The novel small RNA has been termed piYRNA, reflecting size similarity to piRNA and the apparent origin. A northern blot tissue screen indicated piYRNA is located in all tissues examined at similar levels, except for the retina that has > 100 fold higher levels. An affinity pull down method was used to identify piYRNA-binding proteins. This effort found that piYRNA binds several proteins, the most prominent of which was matrin3, a nuclear matrix-associated protein with two zinc finger domains and two RNA binding domains in close proximity. Related studies with piYRNA mutants revealed a significant degree of sequence specificity and that the piYRNA: matrin 3 interaction is contingent upon the RNA being single stranded. Studies with matrin 3 peptides indicate piYRNA binding requires both RNA binding domains (Kd = 20nM). Preliminary experiments indicate that piYRNA interferes with transcription and/or translation, pointing to the possibility that this may represent the physiological mode of action of piYRNA. In summary, these studies have discovered a novel small RNA, piYRNA, that binds to matrin 3 and has the capacity to inhibit transcription and/or translation. The finding of a high concentration of piYRNA in the retina suggests that it might have an important role in this tissue in modulating transcription and or translation.

NICHD
Jovic, Marko
Visiting Fellow
Biochemistry - General and Lipids
Regulation of PI4KIIa Retrograde Transport
Proper function of each cellular compartment relies on maintenance of the unique lipid composition of its membrane. In the Golgi, phosphatidylinositol 4-phosphate (PtdIns4P) is the key regulatory lipid that mediates sorting of proteins out of this compartment. Interestingly, while several PtdIns4P lipid kinases (PI4Ks) localize to the Golgi, a large fraction of PI4KIIa is also found on endosomes. Tight membrane association of PI4KIIa through a palmitoyl moiety implies that this enzyme must continuously cycle between endosomes and Golgi. The importance of PI4KIIa localization is underscored by its role in EGF receptor degradation, Wnt signaling and a late-onset neurodegeneration developing in PI4KIIa gene-trapped mice. Surprisingly, little is known about the molecular events determining the localization and functions of PI4KIIa in various cellular compartments. This study was designed to address the regulation of PI4KIIa transport between Golgi and endosomes. To this aim we performed a proteomic screen of potential binding partners of PI4KIIa using tandem mass-spectrometry analysis of proteins that were co-immunoprecipitated (Co-IP) with PI4KIIa. Intriguingly, among the highest affinity partners was a vesicular fusion protein VAMP-3 that is known to facilitate fusion of endosome-originated membranes with the Golgi. This interaction was confirmed in Co-IP experiments, by performing a pull-down of either VAMP-3 or PI4KIIa followed by blotting against the other binding partner. In control experiments VAMP-3 pull-down did not bring down PI4KIIIb, a PI4K that localizes to the Golgi. Remarkably, expression of Tetanus toxin (TeNT), which cleaves VAMP-3 and inhibits one of the retrograde trafficking routes, resulted in accumulation of PI4KIIa on endosomes as determined by live-cell confocal microscopy. In a control rescue experiment, overexpression of TeNT together with mutant VAMP-3 resistant to TeNT recovered the Golgi localization of PI4KIIa. TeNT will, therefore, serve as a valuable method in our future kinetic studies where a photoactivatable variant of PI4KIIa will be used to assess the rate of its retrograde transport back to the Golgi in the presence of TeNT. Taken together, these findings provided the first evidence of the potential mechanism regulating PI4KIIa retrograde transport, and revealed new approaches by which to study the role of PI4KIIa in cellular processes relevant to human health and disease.

NEI

Subramanian, Preeti
Postdoctoral Fellow
Biochemistry - General and Lipids
Structure-function Relationships of Pigment Epithelium-derived Factor Receptor (PEDFR): Identification of a PEDF Binding Region
Retinal degenerations lead to retina cell death and to irreversible blindness. Pigment epithelium-derived factor (PEDF) is a natural ocular protein with potent retinal survival properties, however, its molecular mechanisms of action remain unknown. We propose that extracellular PEDF acts by interacting on cell surfaces with PEDFR, a novel membrane-linked lipase protein identified in our laboratory. The PEDFR amino acid sequence reveals a patatin-like phospholipase domain near its N-terminus, four transmembrane domains, three intracellular regions and two extracellular loops (L2 and L4). The PEDFR protein exhibits phospholipase A2 (PLA2) activity, and specifically binds PEDF with high affinity resulting in stimulation of fatty acid release from phospholipids. The purpose of this work is to identify a PEDF binding site on PEDFR and its significance for cell survival.
Surface plasmon resonance (SPR), affinity chromatography and pulldown experiments showed that the recombinant catalytic site variant PEDFR[D166A], loop L4 (L159-M325), and C-terminal truncated PEDFR4 (M1-L232) polypeptide fragments bound PEDF with similar affinity as the full length PEDFR (M1-L504) (KD=3-14nM). While the alteration D166A abolished, the PEDFR4 retained PLA2 activity, which PEDF stimulated. We designed synthetic peptides spanning L4 for PEDF binding assays. Ligand blot, SPR and affinity chromatography proved that only peptides E5b (I193–L232), P1 (T210-L249) and E5d (T210-L232) specifically bound PEDF. Moreover, removal of the E5b region from PEDFR and PEDFR4 did not affect PLA2 activity but decreased PEDF-mediated PLA2 stimulation. In experiments with rat retina R28 cells, active PEDFR protein was identified in plasma membranes and PEDF stimulated its activity. Real-time microelectronic cell sensor and cell viability assays showed that PEDF protected R28 from cell death induced by serum starvation in a dose-dependent fashion (EC50=1.6nM). More interestingly, PEDF pre-incubation with molar excess of peptides P1 and E5b attenuated R28 cell survival activity, implying that the peptides act as soluble PEDF receptor fragments that block key PEDF interactions for survival activity. In conclusion, we have identified a novel area within the E5d region of PEDFR polypeptide that contains structural determinants for PEDF binding and PEDF-mediated PEDFR stimulation, critical for cell survival. This constitutes an important advance in elucidating the molecular mechanism of action of PEDF.

NIEHS
Cavanaugh, Nisha
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Biochemistry - Proteins
New Insights into Ribonucleotide Discrimination by DNA Polymerase Beta
Surface plasmon resonance (SPR), affinity chromatography and pulldown experiments showed that the recombinant catalytic site variant PEDFR[D166A], loop L4 (L159-M325), and C-terminal truncated PEDFR4 (M1-L232) polypeptide fragments bound PEDF with similar affinity as the full length PEDFR (M1-L504) (KD=3-14nM). While the alteration D166A abolished, the PEDFR4 retained PLA2 activity, which PEDF stimulated. We designed synthetic peptides spanning L4 for PEDF binding assays. Ligand blot, SPR and affinity chromatography proved that only peptides E5b (I193–L232), P1 (T210-L249) and E5d (T210-L232) specifically bound PEDF. Moreover, removal of the E5b region from PEDFR and PEDFR4 did not affect PLA2 activity but decreased PEDF-mediated PLA2 stimulation. In experiments with rat retina R28 cells, active PEDFR protein was identified in plasma membranes and PEDF stimulated its activity. Real-time microelectronic cell sensor and cell viability assays showed that PEDF protected R28 from cell

death induced by serum starvation in a dose-dependent fashion (EC50=1.6nM). More interestingly, PEDF pre-incubation with molar excess of peptides P1 and E5b attenuated R28 cell survival activity, implying that the peptides act as soluble PEDF receptor fragments that block key PEDF interactions for survival activity. In conclusion, we have identified a novel area within the E5d region of PEDFR polypeptide that contains structural determinants for PEDF binding and PEDF-mediated PEDFR stimulation, critical for cell survival. This constitutes an important advance in elucidating the molecular mechanism of action of PEDF.

NIAID
David, Alexandre
Postdoctoral Fellow
Biochemistry - Proteins
Journey to the Center of Translation: In Situ Visualization and Characterization of Compartmentalized Active Translation Sites
Protein translation is a central cellular function that is the focus of increasing attention as cell biologists integrate gene product-specific information into a systems view of cellular function. Evidence mounts that protein translation is a highly varied process with global adaptations for varied circumstances, including distinct modifications tailored for environmental and infectious stressors and differentiation states. Recent studies further suggest compartmentalization of translation machinery, allowing local translation of specific mRNAs to produce proteins where they are most needed to optimize fundamental mechanisms, such as cell migration, embryogenesis, neuronal synapse formation and viral infection. To better study translational compartmentalization, we developed the RiboPuromyclation method (RPM), which visualizes translating ribosomes in cells by standard immunofluorescence. RPM is based on ribosome-catalyzed puromycylation of nascent chains immobilized on ribosomes by chain elongation inhibitors and detection of the puromycylated nascent chains with a puromycin-specific mAb. Ribosome catalyzed puromycylation occurs in both live and digitonin permeabilized cells and cell extracts. Due to its high sensitivity, RPM enabled us to visualize different aspects of compartmentalized translation. We correlated localized protein synthesis with cell activation and differentiation, stress, and viral infection. Combining cell fractionation and in vitro RPM we identified the first ribosomal sub-population characterized by its peculiar sedimentation in sucrose and its ability to translate a restricted set of proteins. We now intend to purify and identify these nascent chains by mass spectrometry. This study clearly illustrates the strength of RPM as a unique and powerful tool for visualizing and characterizing translation sites that will facilitate understanding of translation compartmentalization across many disciplines in biological research, including eukaryotic and simple prokaryotic cell biology.

FDA/CBER
Fortes de Araujo, Fernanda
Postdoctoral Fellow
Biochemistry - Proteins
Development of a mouse model of Chagas disease and validation of an aptamer- based assay to detect a biomarker of Trypanosoma cruzi infection
The blood borne protozoan parasite Trypanosoma cruzi (T. cruzi) is the causative agent of Chagas disease. This infection is mostly endemic in Central and South America, however, it is estimated that 300,000 persons unknowingly infected with T. cruzi currently reside in the U.S. and several documented cases of T. cruzi transmission by blood transfusion or organ transplantation have been reported in North America. Asymptomatic chronically infected blood donors represent a significant risk to the safety of the U.S. blood supply since these individuals can have fluctuating parasitemia and antibody levels that can be below the limit of detection of the current antibody screening test. In order to study disease

pathogenesis and validate new assays for the detection of a T. cruzi infection either soon after the infection or during the asymptomatic chronic phase of the disease, we have developed a mice model of Chagas disease in our laboratory. In this model C57BL/6 mice are infected with 1,000 parasites of Colombian strain of T cruzi. In this model, infected animals show a rapid increase in parasitemia that peaks at 30 days post infection (dpi). This is followed by a decrease of the number of parasites in blood reaching undetectable levels by microscopy 100dpi. T. cruzi specific IgM and IgG antibodies can be detected 14dpi and IgG levels stay high up the 120 dpi. Nests of parasites can be detected in sections of heart tissues 30 dpi and strong inflammation is observed in the hearts of infected mice 120dpi. These results closely mimic the acute and chronic phases of Chagas disease observed in human infection. This model was used to validate a new Enzyme Linked Aptamer (ELA) assay. In this assay, aptamers (short nucleic acid molecules) developed against T. cruzi excreted/secreted antigens (TESA) are used as parasite specific ligands in an ELISA-like assay. Our results showed that the ELA assay can detect TESA in serum of T. cruzi infected mice as early as 7 dpi as well as during the chronic phase i.e. in situations when parasites are not detectable by microscopy. In conclusion we have developed a mice model that mimics Chagas disease in human. This model was used to validate a novel Enzyme Linked Aptamer Assay that detects a biomarker of T cruzi infection in blood and to show that this assay can be used to detect both early and chronic infections when parasites are undetectable by microscopic methods.

NIAID
Gruber, Todd
Postdoctoral Fellow
Biochemistry - Proteins
A new mechanism for an old drug: the mode of action of the tuberculosis drug p-aminosalicylic acid (PAS)
Tuberculosis (TB) infects approximately one-third of the world's population and is responsible for nearly 2 million deaths per year. Treatment courses require up to four different antibiotics for nine months. Failure to complete the complex treatment regimen has led to widespread instances of drug-resistant strains, necessitating the discovery of new antitubercular agents. We performed a high-throughput screen of 15,000 compounds against Mycobacterium tuberculosis to identify potential drugs. The screening results identified fenamisal, the phenyl ester of p-aminosalicylic acid (PAS). PAS is a widely-used second-line antitubercular drug with an unknown mechanism of action despite usage since the 1940s. Spontaneous TB mutants resistant to fenamisal (and cross-resistant to PAS) were selected and whole genome sequencing revealed point mutations in a single gene, folC, suggesting that fenamisal inhibits this enzyme. We have identified one of these folC point mutations in PAS-resistant patient samples, underscoring the clinical relevance of these results. FolC, also known as dihydrofolate synthase, catalyzes the production of dihydrofolate, the direct precursor of the enzymatic cofactor tetrahydrofolate (THF). We further observed that the biosynthetic step prior to FolC, catalyzed by FolP, uses a substrate very similar to PAS. We hypothesized that PAS acts as a replacement substrate for these enzymes rather than an inhibitor, and that PAS is ultimately incorporated into a PAS-THF derivative which disrupts folate metabolite pools downstream of FolC. We have cloned, expressed, and purified FolC and FolP to study their activity. We found that PAS is indeed an excellent substrate for FolP, and the PAS-derived product is an excellent substrate of FolC. Contrastingly, fenamisal is only a weak substrate for FolP and the fenamisal-derived product is not a substrate for FolC, suggesting that inhibition of FolC is the mechanism of action of fenamisal. We anticipate that the spontaneous mutations observed in FolC eliminate this inhibition and prevent incorporation of PAS; work in this area is ongoing. The discovery of the mechanism of action of PAS will likely result in identification of additional genetic markers of resistance, allowing tailoring of anti-TB regimens for specific patients.

NIAID

Mukherjee, Tathagata
Visiting Fellow
Biochemistry - Proteins
Diagnosis and Treatment of Tuberculosis: One Potential Solution, PA-824
PA-824, a new drug currently in clinical trials for tuberculosis treatment, shows promise for treatment of both active and latent forms of tuberculosis (TB). PA-824 is a nitroimidazole-oxazine pro-drug activated by the deazaflavin dependent nitroreductase (Ddn) encoded by Rv3547 using F420 as the cofactor. The key active metabolite generated from the nitroimidazole group is nitrous acid which disproportionate to form nitric oxide and other reactive nitrogen species. The formation of these reactive nitrogen intermediates correlates with the cidal activity towards non-replicating M. tuberculosis (MTb). We hypothesize that understanding of the enzymatic mechanism will allow us to develop an optimized PA-824 analog with improved bactericidal activity. The X-ray crystal structure for the Ddn enzyme from MTb as well as its active homolog from Norcardia farcinica was solved at less than 2 Å resolutions with or without bound cofactor. Molecular docking in combination with structure activity relationship studies analyzing enzyme kinetics of Ddn identified a number of residues which might constitute the binding pocket of PA-824, which were corroborated by enzymatic analysis of the corresponding site directed mutants. Elaboration of the catalytic mechanism, which involves a novel multistep mechanism consisting of both enzymatic and non-enzymatic transformations, has paved the path towards developing analogs that increase the yield of cidal end products by Ddn. The fact that PA-824 is metabolized by Ddn following uptake by MTb suggests that it might serve as an imaging agent relying on bacteria for localization rather than secondary characteristics of infection, i.e. metabolism or inflammation. To evaluate the potential for PA-824 as a radiotracer, (18F) PA-824 analog has been synthesized for positron emission tomography (PET) imaging studies in MTb-infected and naïve rabbits. The radiotracer appears to concentrate in the granuloma in the rabbit lung (2-3-fold signal increase compared to normal lung tissue) thereby; highlighting its potential not only in diagnosis of TB but also the evaluation of treatment response as the bacterial burden decreases.

NCI-CCR
Nguyen, Giang
Doctoral Candidate
Biochemistry - Proteins
High-throughput identification of compounds targeting the human Bloom protein
Bloom protein (BLM) is a member of the RecQ DNA helicase family with a unique ability to unwind a variety of DNA structures that arise during DNA replication and repair. BLM has been implicated in many cellular processes, particular pathways involved in maintaining genome integrity. Mutation of BLM leads to Bloom syndrome (BS), a condition that has an increased predisposition to cancer, growth retardation, immunodeficiency, sunlight sensitivity and infertility. Despite an extensive characterization of BLM in cellular processes and pathological conditions, further analysis of BLM has been severely hampered by the fact that BLM is tightly cell-cycle regulated and that BLM null mice showed embryonic lethality. Therefore, a BLM-specific inhibitor that can be readily applied to cells and in animal models will become an invaluable tool for the research community. We now report the discovery of the first potent and selective cell-active small molecule inhibitor for BLM known as NIH505 (IC50 = 5.6µM). This inhibitor was identified from the NIH small molecule repository library of 320,000 compounds using a human BLM-based fluorescence polarization high throughput screening assay. NIH505 inhibited BLM unwinding a variety of DNA substrates with different structures and length ? forked duplexes, Holliday junction, displacement loop, duplexes with internal “bubbles”, and G4-duplexes ? at 5.5-10.2µM. Inhibition by NIH505 was BLM-specific, because other RecQ helicases ? RecQ1, RecQL5, WRN, and UvrD ? were not significantly inhibited. Moreover, NIH505 was not competitive with ATP and targeted BLM’s DNA binding

region. In cell-based assays, NIH505 abrogated many BLM-mediated cellular functions. Like BS cells, wild-type cells treated with NIH505 showed slow-growth phenotype, higher sensitivity to DNA-damaging agents, an approximately threefold increase in the frequency of sister chromatid exchange, and endogenously activated DNA double-strand break checkpoint response with prominent levels of phosphorylated ?-H2AX in nuclear foci compared to DMSO treated cells. Interestingly, NIH505 inhibited the proliferation of tumor cells that maintain stable telomere length by the alternative lengthening of telomere pathway compared to telomerase-positive cells, suggesting that NIH505 may have a new life as a cancer drug. In summary, our findings indicate that NIH505 is a potent, specific and selective BLM inhibitor with valuable pharmacological and cell biological potential.

NIDDK
Anthis, Nicholas
Postdoctoral Fellow
Biophysics
Global Regulation of the Structure, Dynamics, and Function of the Calcium Sensor Protein Calmodulin Revealed by Paramagnetic NMR
As the cell's prototypical calcium sensor, calmodulin (CaM) is responsible for amplifying and adapting this universal signal into specific downstream signals via interactions with hundreds of other proteins. For many of these targets, activation occurs when calcium-loaded CaM relieves self-inhibition by binding to a regulatory peptide. CaM consists of two domains separated by a central linker, and although crystallographic studies have consistently found peptide-free CaM in an extended "dumbbell" conformation, NMR has demonstrated that the central linker is actually quite flexible. Calcium alters the structure of each domain, exposing hydrophobic patches for peptide binding. Calcium-loaded CaM can then interact with a variety of targets, forming a much more compact structure. These changes require significant conformational flexibility, but little is known about how these motions directly contribute to biological function, and what role sparsely populated minor states may play. Although such minor states are inaccessible to traditional structural methods, new developments in paramagnetic relaxation enhancement (PRE) have made it possible to study these states. Because its sharp distance dependence (r^-6) leads to extremely large PREs at short distances, the footprint of a minor species can be observed in the overall PRE profile if the distances involved are shorter in the minor species than in the major one. We have measured PREs for CaM with and without calcium, in peptide-bound and unbound states, with paramagnetic spin labels attached to either domain. The intradomain PREs could be easily fit to known structures, but only in peptide-bound CaM could the interdomain PREs be fit. Fitting the interdomain data from unbound CaM required generating an ensemble of structures using simulated annealing calculations in which the central linker was allowed to be flexible for a minor fraction of the population (~10%). In these cases, CaM samples a wide range of structures, but calcium causes it to favor closed states resembling the peptide-bound structure. Calcium-free CaM does not adopt these closed structures, even in the presence of peptide. These findings imply that calcium activates CaM through more global effects, with calcium allowing CaM to clamp down upon its peptide target in a manner not possible in the apo state. Thus, PRE has given us novel insight into this biological process of central importance.

NICHD
Banerjee, Anand
Postdoctoral Fellow
Biophysics
Mathematical modeling of clathrin mediated endocytosis

Clathrin mediated endocytosis (CME) is a process by which eukaryotic cells internalize various macromolecules. It is of fundamental importance to organisms in a number of ways, including nutrition, cholesterol metabolism, hormone responsiveness, and the passive acquisition of immunity during fetal and neonatal development. CME starts with the assembly of a protein coat on the inner membrane of the cell, which leads to the formation of small invaginations called clathrin coated pits (CCPs). After recruiting cargo, a CCP grows in size and invaginates further, until it is joined to the membrane by a narrow neck. Finally, the neck is pinched off and the cargo-containing clathrin coated vesicle (CCV) is taken into the cell for processing. Although the molecular players involved in CME are well recognized, the kinetics of CCP assembly is still poorly understood. The reason is that complete understanding of CCP assembly requires coupling of information on protein-protein and protein-lipid interactions with a description of the mechanics of the cell membrane and protein coat deformation. Studies on CME so far have not attempted to account for this coupling. To address this problem, we model CCP assembly as a nucleation phenomenon. The inputs to our model are the protein-protein and protein-lipid association/dissociation rate constants, and the free energy of formation of CCPs. We obtain the rate constants from biochemical experiments, and calculate the free energy using elasticity theory. Our results show that the formation of a CCV from a CCP can be understood as a phenomenon where a CCP of small size crosses a free energy barrier in order to grow and form a CCV. In the absence of cargo, the free energy barrier is too high and most of the CCPs are abortive, i.e., they grow only partially and then disassemble. The model accurately reproduces the statistical properties of abortive pits, such as their lifetime and size distribution. We also find that the free energy barrier is lowered in the presence of cargo, so a CCP can transform into a CCV. Our model combines detailed molecular level information with the shape and mechanical properties of a CCP to build an integrated theory of CME. It provides insights which are not obtainable through biochemical experiments alone, and therefore is a complimentary and significant step towards fully understanding CME.

NIDCD
Gavara, Nuria
Postdoctoral Fellow
Biophysics
Being out of shape. Cell stiffness correlates with actin fiber content rather than cell area.
Recent studies stressing the effect of geometric cues on cell differentiation and contractility have brought new interest to the role of cell shape in cellular function. In particular, cell spreading is believed to increase cell stiffness via upregulation of cytoskeleton (CSK) contractility. Previous studies aimed at correlating cell stiffness with simple parameters that could be easily measured, such as cell spreading, disregarding others like actin fiber content. This was in part due to the difficulty of measuring cell stiffness and actin fiber content simultaneously in living cells. We have combined live-cell imaging of GFP-actin transfected cells, novel image processing algorithms and Atomic Force Microscopy (AFM) to assess for the first time the relationship between cell spreading, actin fiber content or organization, and cell stiffness. Cell area (A) was measured by detecting the cell contour from fluorescence images. Actin fiber content (F) was measured by dissecting out actin fibers from the overall GFP signal and quantifying the fiber's fluorescence intensity (as arbitrary units; AU). Actin organization was measured by computing the angular direction of the actin fibers. Cells were sorted out as having aligned fibers (angular variance<0.25) or randomly-oriented fibers (angular variance>0.4). Cell stiffness was measured by indenting several areas of the cell with an AFM tip and computing Young's modulus (E in kPa). Cell stiffness increased linearly with actin fiber content (E=aF) for both types of fiber organization, but slopes were significantly different (a=1.25 kPa/AU for aligned, a=2.82 kPa/AU for random; P=0.0002 analysis of covariance test). A CSK with randomly-oriented fibers has more intersecting points between fibers and is likely to be more crosslinked, which provides further mechanical strength to a structure. Increased

numbers of crosslinks can thus explain the larger slope we found for randomly-oriented CSKs. We also investigated which parameter, cell area or fiber content correlates better with cell stiffness. When we fitted E=ßA, residuals were larger than for E=aF (res=2313 for EvsA, res=1707 for EvsF; P=0.33 F-test for equality of variances), which suggests that cell stiffness correlates with actin fiber content rather than cell area. Similar experiments are planned with GFP-myosin and GFP-a-actinin. Our results reveal the key players on cell mechanics and have implications for the field of cell mechanosensing.

NIDDK
Rosta, Edina
Research Fellow
Biophysics
Two-metal Ion Catalysis of RNA Backbone Cleavage by Ribonuclease H
Phosphate esters are essential to living systems. In a large number of enzymes, the phosphate ester hydrolysis is catalyzed by two bound divalent metal ions. However, the detailed mechanistic steps of the two-metal ion catalysis reaction are not yet known and understood. We recently found that coupled proton transfer reactions and metal coordination emerge as central factors in the catalytic RNA cleavage reaction. However, the exact roles of the two metal ions remains unclear. We carry out hybrid quantum-classical QM/MM free energy simulations of the catalytic reaction of the bacillus halodurans RNase H enzyme complexed with an RNA/DNA hybrid substrate. RNase H is a prototypical member of a large family of enzymes that use two-metal ion catalysis to process nucleic acids. The active site of RNase H is almost identical across species with respect to sequence and structure, including the human and the HIV Reverse Transcriptase (HIV-RT) RNases H. The HIV-RT is essential to viral replication, which makes it a main target in HIV drug research. We combine Hamiltonian replica exchange with a finite-temperature string method, to calculate the accurate QM/MM free energy surface underlying the catalytic reaction. We also derive new histogram-free equations for estimating the unbiased free energy from combined multidimensional string simulations. Our method allows us to search for the optimal pathway in multiple dimensions and, therefore, to identify the exact sequence of the underlying steps of the RNA cleavage reaction. We find that the presence of both catalytic Mg ions is an essential requirement for catalysis. Replacing either of them with a Ca ion abolishes the catalytic activity. Double Mn or Ca ion replacements have been characterized experimentally and our calculations agree well with measured catalytic activities. Moreover, single ion replacements, which can only be performed in our simulations, demonstrate that the main role of the metal ions is to decrease the pKa of the oxygen nucleophiles and, therefore, to decrease the barrier of the coupled proton transfer processes governing the catalytic reaction. Our new proton transfer mechanism is consistent with kinetic effects of protein mutations and RNA backbone modifications. Moreover, the highly accurate transition state structures are an ideal target for future structure-based drug design studies of new HIV-specific inhibitors.

NICHD
Zustiak, Silviya
Postdoctoral Fellow
Biophysics
Hindered Diffusion in Polymeric Solutions Studied by Fluorescence Correlation Spectroscopy
The typical cell is crowded with both charged and neutral molecules which hinder solute diffusion and ultimately impact all cellular processes. For example, crowding has been shown to trigger aggregation of the amyloidogeneic proteins associated with neurotoxicity in Alzheimer's disease. Despite the vast interest in the subject, hindered diffusion in the cell is still poorly understood. In particular, the combined effect of crowding and non-specific binding has rarely been addressed, partly due to the inability to decouple their individual contributions in the heterogeneous cell environment. We

developed a homogeneous in-vitro cell model, with tunable binding and crowding, in order to elucidate their relative roles in solute diffusion. We used Fluorescence Correlation Spectroscopy (FCS) to measure the diffusion of a charged protein, Ribonuclease A (RNase), in dextran solutions of various charges (acting as binders) and concentrations (acting as crowders). Originally designed for measurements in dilute media, FCS has recently been employed in studies of diffusion in crowded media. However, such diffusion is mainly interpreted as anomalous, with little insight into the specific causes for the anomaly. We used a more complex two-component FCS analysis, which allowed us to extract quantitative binding data. The non-specific nature of the binding was confirmed by addition of salt, which prevented binding by shielding the RNase and dextran charges. Moreover, we validated our FCS results by a separate, well established technique - ultrafiltration. In agreement with existing data, we observed an overall 5 fold decrease in RNase diffusivity at the highest concentration of dextran, where binding accounted for 75% and crowding for 25% of the decrease. Interestingly, binding decreased RNase diffusivity by 32% even at 0.4 µM dextran. In contrast, crowding affected diffusivity only above a crowder concentration of 20 µM. Further analysis revealed that 100 µM crowder, as compared to 1 µM binder, was needed to achieve equivalent reduction in RNase diffusivity. However, the data suggested that at a higher crowder concentration (300 µM, similar to that in the cell), crowding would overpower the effect of binding. This is the first study to highlight the relative contribution of non-specific binding and crowding to hindered diffusion in crowded and charged media and thus can facilitate future understanding of molecular transport implicated in key cellular processes.

NCI-CCR
Achyut, Bhagelu
Postdoctoral Fellow
Carcinogenesis
Stromal deletion of TGF-ß signaling promotes inflammation-induced squamous cell carcinoma
Deletion or attenuation of transforming growth factor beta (TGF-ß) signaling has significant impact on human carcinogenesis. In addition, inactivation of tumor suppressor genes (p53/PTEN) in stroma results in epithelial cancer (prostare/mammary) development, suggesting an important role of stroma in epithelia homoeostasis. Specific deletion of Tgfbr2 gene in a subset of fibroblast (Tgfbr2fspKO, cre expression under fibroblast specific protein 1 promoter), leads to development of squamous cell carcinoma (SCC) in the forestomach of 100% Tgfbr2fspKO mice. Here we investigated the underlying mechanisms for SCC development. We first confirmed that Tgfbr2 deletion is restricted to the stromal compartment but not epithelial cells using TßR2 immunofluorescence, ß-galactosidase immunohistochemistry in FSP-Rosa26 reporter mice, and phosphorylation of smad2, downstream mediator of TGF-ß signaling. We found that stromal deletion of Tgfbr2 resulted in a significantly increased inflammation, featured by increased CD45+ leukocyte infiltration as early as three weeks but the degree of inflammation was elevated in 4 to 5 week-old mice. The significantly elevated expression of inflammatory mediators such as iNOS, COX2 and NFkB was observed in both epithelial and stromal compartment of forestomach in Tgfbr2fspKO mice. Inflammation is a well known factor that causes DNA damage. We next stained the tissues for ?-H2AX, a histone protein known to be elevated in DNA double strand break, and 8-Oxo-dG, a DNA adduct due to oxidation. We noticed a clearly positive staining for ?-H2AX and 8-Oxo-dG, in both stroma and epithelia. To further look into whether inflammation plays a causal role in the SCC development, we treated Tgfbr2fspKO mice with an iNOS inhibitor (L-NAME). L-NAME significantly reduced the nitrogen oxide production and increased survival (p<0.001) compared to untreated Tgfbr2fspKO mice. L-NAME treatment delayed epithelial hyperplasia/dysplasia, and reduced DNA damage in Tgfbr2fspKO mice as compared to untreated Tgfbr2fspKO mice. Further, we examined human esophageal cancers and observed a downregulation of TßR2 in the stromal fibroblast, and an increased inflammation and DNA damage. Our data suggest that deletion of Tgfbr2 gene in a

subset of fibroblast induces inflammation and DNA damage that play a causal role in SCC development. Therefore, therapeutic targeting of inflammation may be useful in treating cancers with downregulation of TGF-ß signaling in stroma.

NCI-CCR
Chavez, Kathryn
Postdoctoral Fellow
Carcinogenesis
Drozitumab, a human agonist monoclonal antibody to TRAIL-R2 induces apoptosis in triple-negative breast cancer cell lines with mesenchymal features.
Clinically, breast cancers can be divided into distinct subtypes that express estrogen receptors (ER), progesterone receptors (PR), those that have amplification of HER2/Neu, and those that lack expression of ER or PR and lack amplification of Her2/Neu (so called triple negative breast cancer or TNBC). TNBC has a poor outcome compared to the other subtypes of breast cancers. Previously, we have shown that a recombinant GST fusion protein with TNF-related Apoptosis Inducing Ligand (GST-TRAIL) selectively kills triple-negative breast cancer cells with mesenchymal features by activation of TRAIL receptor 2 (TRAIL-R2). In this study we have characterized the growth inhibitory effects of a clinically relevant agonistic antibody to TRAIL-R2, referred to as Drozitumab. To determine the selectivity of Drozitumab, we chose a panel of 15 breast cancer cell lines including 3 ER/PR positive cell lines, 4 HER2/Neu amplified cell lines, and 8 triple-negative cell lines. Treatment of these cells with Drozitumab selectively killed TNBC cell lines with mesenchymal features. ER positive, HER2/Neu amplified and TNBC cell lines with epithelial features were resistant to Drozitumab induced cell death. Drozitumab induced caspase activation (measured by activation of the initiator caspase 8, activation of the downstream caspases 3/7, and PARP cleavage) and the toxicity of Drozitumab was blocked by the pan-caspase inhibitor z-VAD-FMK. This was identical to the pattern seen with GST-TRAIL. Cross-linking with an anti-FC antibody enhanced the efficacy of Drozitumab as shown by the more rapid appearance of cleaved caspase 8, greater caspase 3/7 activity, and more rapid PARP cleavage. This data provides evidence that TRAIL-R2 targeted therapies may have therapeutic potential in the treatment of TNBC with mesenchymal features.

NCI-CCR
Hudson, Robert
Postdoctoral Fellow
Carcinogenesis
The microRNA-106b-25 cluster increases the oncogenicity of human prostate cells and directly targets Caspase-7
Previously, we found several miRNAs from the polycistronic miR-106b-25 to be elevated in prostate cancer, consistent with an oncogenenic role for this miRNA cluster. Additionally, this cluster is a homolog to the prototypical oncomiR-1 (miR-17-92) and is thought to act as a super-oncogene when co-amplified with its host gene minichromosome complex maintenance component 7 (MCM7). We hypothesize that the expression of miR-106b-25 acts as a transforming factor in prostate cancer. To examine this, we first determined if miR-106b-25 could contribute to the malignant transformation of prostate cells and then investigated novel targets for miR-106b. To begin, we preformed xenografts in nude mice with normal immortalized non-tumorigenic RWPE-1 prostate cells infected with lentivirues engineered to confer the overexpression of the MCM7 intron that houses the complete miR-106b-25 gene. These studies showed a significant increase in tumor formation and growth within animals inoculated with miR-106b-25 over-expressing cells when compared to control groups. To explore the mechanism and potential targets of miR-106b specifically, we transiently transfected prostate cancer

LNCaP cells with oligo mimics of mature miR-106b and performed mRNA microarray analysis. We found that transcripts that were down-regulated in response to miR-106b were significantly enriched for miR-106b predicted targets. In addition, miR-106b altered transcripts were enriched for cell death pathways genes, suggesting a possible role of miR-106b in the apoptotic response of prostate cancer cells. Among the most highly down-regulated transcripts associated with miR-106b was that of Caspase-7 (CASP7), a central executioner of apoptosis. Western blot analysis in RWPE-1, 22Rv1, and LNCaP prostate cells confirmed that miR-106b could reduce CASP7 protein levels. Using target prediction algorithms we identified a highly conserved miR-106b seed site within the 3'UTR of CASP7. Direct targeting of the CASP7 3'UTR by miR-106b was confirmed using wild-type and miR-106b seed site mutant 3'UTR luciferase reporter assays. This research reveals a novel oncogenic role for the miR-106b-25 cluster in prostate cancer and, for the first time, indentifies CASP7 as a direct target of miR-106b. Future work is aimed at understanding the role of CASP7 in the function of miR-106b and the possible utility of targeting miR-106b as novel therapy for prostate cancer.

NCI-CCR
KIM, DONGWOOK
Postdoctoral Fellow
Carcinogenesis
Mutation of Thyroid Hormone Receptor ß in Mice Predisposes to the Development of Mammary Tumors
Thyroid hormone receptors (TRs) are ligand-dependent transcription factors that mediate the biological activities of thyroid hormone (T3) in growth, differentiation and development. Correlative data suggest mutations of TR subtype ß (TRß) could increase the risk of mammary tumor development, but unequivocal evidence is still lacking. To explore the role of TRß mutants in vivo in breast tumor development and progression, we took advantage of a knock-in mouse model harboring a dominant negative mutation in the Thrb gene (ThrbPV mouse) that we have developed. The TRßPV mutation was identified in a patient with resistance to thyroid hormone. It has a frameshift mutation in the C-terminal 14 amino acids in TRß, resulting in the complete loss of T3 binding and transcription activity. In adult nulliparous females, a single ThrbPV allele did not contribute to mammary gland abnormalities, but the presence of two ThrbPV alleles led to mammary hyperplasia in ~36% of ThrbPV/PV mice. The TRßPV mutation further augmented the risk of mammary hyperplasia in a mouse model with high susceptibility to mammary tumors (the Pten+/- mouse), as demonstrated by the occurrence of mammary hyperplasia in ~60% of ThrbPV/+Pten+/- and ~77% of ThrbPV/PVPten+/- mice versus ~33% of Thrb+/+Pten+/- mice. The activity of signal transducer and activator of transcription (STAT5), known to play a critical role in mammary tumor development, was increased by TRßPV in ThrbPV mice, as evidenced not only by western blot analysis, but also by immunohistochemistical analysis, showing an increased phosphorylated STAT5 (phosphorylated tyrosine 694/699). The activation of STAT5 by increased phosphorylation led to increased cell proliferation. To understand the molecular mechanism underlying STAT5 overactivation by TRßPV, cell-based studies with T47D breast cancer cells treated with T3 repressed STAT5 signaling in TRß-expressing cells through decreasing STAT5-mediated transcription activity and target gene expression, whereas sustained STAT5 signaling was observed in TRßPV-expressing cells. Collectively, these findings show for the first time that TRß mutations could promote the development of mammary hyperplasia via aberrant activation of STAT5. Moreover, the study also shows that TRß mutants could collaborate with key regulators (e.g. loss of PTEN) to drive breast tumorigenesis. Importantly, the present study has uncovered a novel oncogene that contributes to mammary tumorigenesis.

NIDDK
VAZQUEZ-ORTIZ, GUELAGUETZA

Visiting Fellow
Carcinogenesis
Drug repurposing strategy identifies novel antitumoral molecules for BRCA1 associated breast cancer treatment that are synergistic with resveratrol
About 8% of breast cancer cases are inheritable, caused by mutations in some genes such as breast cancer associated gene 1 (BRCA1). Our preliminary data showed that resveratrol induced stronger cell death in BRCA1 mutant (mt) cells with respect of BRCA wild-type (wt) in vitro and in vivo. Drug repurposing consists of the process of developing new applications for existing drugs, therefore in order to identify small molecules that can be repurposed and synergize the killing effect of Res in breast cancer cells we created a pair of isogenic breast cancer cell lines (92J-shBRCA1 and 92J-wt derived from MBA-MD-231) and used them to screen the NCGC Pharmaceutical Collection (NPC). The NPC consists of 2816 small molecule compounds of which are approved for human or animal use by FDA or other countries. Using a high-throughput ATP-release assay, we identified the inhibitory effect of 43 compounds that exhibited synergy with Res with efficacy>50% and potency 35uM. The initial screening was followed by a validation using the same approach and same drugs with additional pair of isogenic breast cancer cells from MBA-MD-231 and a series of validations including MTT and ATP-release with drugs obtained from different vendors. The effect was confirmed in 11 compounds. The combination of cantharidin, chloroquine, amodiaquin and colchicine with Res is successful by killing preferentially BRCA1-mt cells in vitro. On the other hand, Narasin and Laureth-4 combined with Res killed BRCA1-wt better. Studies utilizing BRCA1-mt breast tumor xenograft models in aythymic mice reveal that mice treated with combination therapy exhibit significantly reduced tumor volumes and increased survival compared with mice on monotreatment. Our further study revealed that cantharidin, chloroquine, amodiaquin and colchicine target the mTOR/AKT pathway at different levels and produce strong vacuolization in the cytoplasm during treatment, deregulating macro-autophagy, followed by an induction of apoptosis on the cells. Furthermore, a kinase assay using cantharidin as treatment revealed a downregulation in the activity of PP2A (directly involved in mTOR/AKT pathway) and downregulation in the expression of a recently described proto-oncogene AXL. RNAi and pharmacological inhibition of AXL inhibited migration and metastasis in vitro and in vivo in BRCA1-mt cells. This approach offers the potential to identify new purposes for old drugs to treat BRCA1 associated breast cancer.

NIEHS
Yin, Zhengyu
Visiting Fellow
Carcinogenesis
RAP80 Plays a Critical Role in Maintaining Genomic Stability and Tumor Suppressing
The DNA damage response (DDR) coordinates activation of cell cycle checkpoints, apoptosis and DNA repair networks, to ensure accurate repair and genomic integrity. Phosphorylation of the histone H2A variant, referred as gamma-H2AX, is one of the initial signaling events which sense DNA double strand breaks and is required for the subsequent recruitment of many DDR proteins to sites of DNA damage. Upon DNA damage by ionizing irradiation (IR), the ubiquitin interaction motif (UIM)-containing protein RAP80 binds to poly-ubiquitin chain of H2A and gamma-H2AX, and mediates DNA repair events by recruiting DDR mediators and effectors, especially BRCA1. Knockdown of RAP80 expression in vitro greatly impairs the localization of BRCA1 to the IR-induced foci and therefore increases susceptibility to IR or other DNA damaging agents. Based on these observations, we hypothesized that RAP80 functions as a tumor suppressor gene and that deficiency in RAP80 may lead to genomic instability in vivo and promote cancer. In this study, RAP80 knockout (KO) mice were generated and characterized. In contrast to the embryonic lethal phenotype in BRCA1 KO mice, RAP80 KO mice are viable and do not exhibit any major anatomical defect. However, we showed that mouse embryonic fibroblasts (MEFs) from RAP80

KO mice exhibited slower rate of proliferation as well as a higher percentage of premature senescence compared to wild type (WT) MEFs. RAP80 KO MEFs also showed increased spontaneous and IR-induced genomic instability, which led to prolonged G2/M cell cycle arrest. Moreover, RAP80 KO MEFs contained a higher percentage of spontaneous gamma-H2AX positive cells compared to WT MEFs and IR induced more nuclear fragmentation in RAP80 deficient MEFs than WT controls. Loss of RAP80 increased sensitivity to IR in vivo and in vitro while IR-induced gamma-H2AX foci disappeared at a slower rate in RAP80 KO MEFs. In addition, in response to IR, p53 and several p53 targeting pro-apoptotic genes were activated in absence of RAP80. Tumorigenesis studies suggested that RAP80 KO mice exhibited an increased susceptibility to the spontaneous development of lymphoma and the development of DMBA-induced mammary gland cancer. Altogether, these data indicate that RAP80 functions as a tumor suppressor gene and that deficiency of RAP80 leads to genomic instability and predisposition to cancer.

NCI-CCR
Afonso, Philippe
Postdoctoral Fellow
Cell Biology - General
Leukotriene B4 autocrine/paracrine secretion amplifies fMLP-gradient sensing during neutrophil chemotaxis.
Neutrophils have the capacity to chemotax, i.e. sense and migrate directionally toward gradients of external chemicals. Usually chemotaxis is studied at the single cell level, with a focus on the different intracellular pathways activated by a given chemoattractant. We wanted instead to analyze the migration of neutrophils at the cell population level where multiple chemoattractants ultimately dictate directed migration. Indeed, in response to primary chemoattractants, such as the bacterial peptide fMLP, neutrophils secrete the secondary chemoattractant leukotriene B4 (LTB4). We hypothesize that LTB4 autocrine/paracrine secretion plays an important role when neutrophils migrate towards the primary chemoattractant fMLP. For this purpose, we used drugs that block LTB4 signaling (MK886, inhibits leukotriene production, and LY223982, a LTB4-receptor antagonist) and assessed their effects on primary human neutrophil activation and chemotaxis. While we show that treatment of neutrophils with either drug has no impact on cell activation following high concentrations of fMLP, we find that LTB4 production is important for intracellular cAMP accumulation, actin polymerization and cell polarization when cells are stimulated with a low concentration of fMLP. We also show that the inhibition of LTB4 relay inhibits chemotaxis to fMLP. Interestingly, we establish that the inhibition is stronger when cells migrate towards lower, more physiological, concentrations of fMLP. Remarkably, LTB4 signaling did not impact fMLP-mediated activation of either Akt/PKB or ERK. Finally, we demonstrate that neutrophils isolated from mice lacking the formyl receptor 1 (FPR1) can migrate to fMLP when mixed with neutrophils isolated from WT mice, as these latter secrete LTB4 which allows recruitment of FPR1-/- neutrophils. Together, our findings demonstrate that signal relay plays a key role during neutrophil chemotaxis. We propose that LTB4 secretion following fMLP stimulation forms a secondary gradient that significantly amplifies the overall neutrophil population recruitment to primary chemoattractants. Importantly, we show that the LTB4 gradient is more important as neutrophils migrate to lower/physiologically relevant gradients of primary chemoattractants. We envision that LTB4 mediates these effects by selectively activating a subset of effectors.

NICHD
Elia, Natalie
Postdoctoral Fellow
Cell Biology - General

Spatial and temporal resolution imaging of the ESCRT machinery in cytokinesis: defining an ordered mechanism in mediating the final cut.
The final stage of cytokinesis is abscission, the cutting of a thin microtubule based bridge connecting the two daughter cells. Regulation of abscission is thought to take place at the midbody, a highly dense structure at the center of the microtubule bridge. The ESCRT components have been previously localized to the midbody and their midbody localization was crucial for proper cytokinesis. However, their role in abscission has not been identified. Using structured illumination microscopy we have mapped the localization of the different ESCRT components at the midbody. We found that TSG101 (ESCRT-I) forms a double ring structure at the center of the midbody where it binds to its partner CEP55. The ESCRT-III component CHMP4B was recruited to that ring but at late stages of abscission also co-localized with a narrow constriction zone precisely at the site of cutting. High sensitive time lapse microcopy provided direct visualization of the kinetics and the ordered assembly of the ESCRT components at the midbody. CEP55, arrives to the midbody early at cytokinesis. TSG101, the ESCRT-I components that binds to CEP55, arrives later and shows a continuous recruitment until the time of abscission. The ESCRT-III component, CHMP4B, follows TSG101 but in addition show acute recruitment very close to abscission. The ESCRT disassembly factor, VPS4 has similar kinetics to that of CHMP4B but its peak is even closer to abscission indicating its function downstream to ESCRT-III. The acute recruitment of both CHMP4B and VPS4 was not to the center of the midbody but rather to the site of narrow constriction and was followed by an acute constriction of the microtubule bridge. These results demonstrate, for the first time, the ordered assembly of the ESCRT machinery in living cells and propose a direct role for these proteins in abscission.

NIA
Ghosh, Avik
Visiting Fellow
Cell Biology - General
RECQL4, the protein mutated in Rothmund-Thomson syndrome, functions in replicative maintenance of telomeres
Telomeres are structures at the ends of linear eukaryotic chromosomes and composed of long tracts of short tandem repeat DNA sequences and telomere specific DNA binding proteins (shelterin). The highly repetitive G-rich sequences at the ends of telomeres can adopt unusual DNA secondary structures, such as G-quadruplexes and D-loops and thus presents a challenge to DNA replication. While RecQ helicases WRN and BLM has been widely implicated in resolving these structures and thus facilitating telomere metabolism, very little is known about the RecQ helicase mutated in Rothmund-Thomson syndrome (RTS), RECQL4. Here we have studied the role of RECQL4 in telomere maintenance. To determine the effects of depletion of RECQL4 on telomeres, immunoflurescence and fluorescence in situ hybridization studies were performed. These studies revealed that, in replicating human cells transduced with RECQL4-targeted shRNA the DNA damage response protein 53BP1 was activated and the frequency of telomere sister chromatid exchanges and fragile telomeres increased, relative to the control cells. The frequency of fragile telomeres was also elevated in RTS patient fibroblasts compared to age and sex matched control fibroblasts, indicating that defects in telomere maintenance in absence of a functional RECQL4. Additional evidence that RECQL4 plays a role in telomere biology was obtained from immunofluorescence which showed that RECQL4 localized to telomeres during replication and co-localized with the shelterin proteins, TRF1 and TRF2. These two proteins also stimulate RECQL4 helicase activity on telomeric D-loop DNA substrates in vitro. RECQL4 could also be co-immunoprecipitated with TRF2 from cell extracts. Further, RECQL4 colocalizes with WRN, another RecQ helicase that plays a role in telomere maintenance, and interacts functionally with WRN during telomeric D-loop unwinding. Thus, we propose that RECQL4 have specialized functions in replication of telomeres, possibly through

resolution of unique telomeric DNA configurations. Our data provides the first instance of interaction between two RecQ helicases in telomere maintenance and contribute towards significant conceptual advances in the RecQ helicase field and as well as in general telomere biology studies. Additionally, this work also contributes towards better understanding of RTS disease pathology, as to our knowledge this is the first report of a telomere defect in a RTS patient cell.

NCI-CCR
Salton-Morgenstern, Maayan
Visiting Fellow
Cell Biology - General
Identification of epigenetic regulators of alternative pre-mRNA splicing
Splicing of precursor mRNA (pre-mRNA) is an important regulatory step in gene expression. Alternative splicing (AS) allows the production of multiple protein isoforms from one pre-mRNA molecule, thereby contributing to proteomic diversity. Novel high-throughput sequencing technology has recently revealed that more than 90% of human genes undergo AS and AS is crucial in differentiation and tissue-specific gene expression. Importantly, mutations affecting AS account for up to 20% of human disease mutations. Cellular signals or environmental stimuli can determine the outcome of AS through trans-acting splicing regulatory factors. Despite the importance of AS, the question of how mRNA splicing machinery identifies the precise location of short exons within vast introns and how AS decisions are made, particularly in a cell type- and tissue-specific fashion, remains to a large extent obscure. Recent evidence points to the fascinating possibility that epigenetic marks and higher order chromatin structure may play a key role in AS regulation. In order to identify novel chromatin regulators of AS, we have developed a cell-based in-vivo assay for high-throughput screening. The assay consists of a two-color fluorescent reporter based on alternative splicing of the microtubule-associated protein TAU, whose mis-splicing cause frontotemporal dementia. This well characterized reporter allows us to identify novel universal regulators of alternatively splicing and at the same time discovering new players in TAU splicing. We have adapted the assay to a 384-well format and have screened a siRNA library of ~400 chromatin-related proteins using high-throughput imaging. The screen identified 18 novel AS candidates. All candidates shift the splicing of the TAU reporter in a way that would correct the splice anomaly in frontotemporal dementia. Thus our candidates are not only shedding light on the emerging role of epigenetic regulators in the process of AS, but also have the potential to define new therapeutic directions for frontotemporal dementia.

NICHD
Sengupta, Prabuddha
Visiting Fellow
Cell Biology - General
NANO-SCALE SPATIAL ORGANIZATION OF PLASMA MEMBRANE REVEALED BY PAIR-CORRELATION ANALYSIS
TIRF-based photoactivated localization microscopy (PALM) offers a powerful tool to interrogate the spatial distribution of proteins in the plasma membrane (PM) at scales inaccessible by conventional light microscopy. We employed PALM to map out the nanoscopic organization of PM and construct a mechanistic perspective of PM organization. Cluster analysis of PALM data is complicated by the multiple appearances of a single protein with associated stochastic uncertainty in position, and reversible blinking of individual fluorophores. We developed a statistical algorithm to rigorously analyze PALM-data by performing spatial pair auto- and cross-correlation on the entire ensemble of detected molecular peaks, and subsequently separating out the contribution resulting from multiple appearances of the same molecule from that arising due to the actual lateral organization of the protein molecules.

Using this approach, we nanoscale protein organization can be described in an accurate and quantitative way, and physical parameters like cluster-radius, average number of proteins per cluster, and the degree of clustering can be evaluated. To use our analysis to validate and extend the existing ideas about PM protein lateral organization, we examined the nanoscale lateral organization of a diverse set of proteins with distinct membrane anchors and partitioning preferences in biochemical membrane preparations. Correlation analysis of high-density PALM images reveal distinct organization principles: Transferrin Receptor is organized into clusters of 100-200 nm, while Vesicular Stomatitis Virus Glycoprotein molecules are present as trimers. PAGFP-GPI and Lat, though grouped together as order-preferring proteins by biochemical criteria, show distinct organizational features: PAGFP-GPI is organized into densely packed clusters of ~5 proteins whereas Lat-PAGFP exhibited clustering along a wide range of spatial scales(50-200 nm). Interestingly, cross-correlation of 2-color PALM images showed dramatic reorganization of actin into ring-like structures around antibody-crosslinked PAGFP-GPI clusters; similar actin-structures can modulate protein-platforms during cell-signaling. Since PALM enables the interrogation of a broad range of spatial scales (~50-250 nm) along which plasma membrane reorganization happens during various physiological processes, we believe this approach will prove valuable in obtaining a comprehensive, mechanistic understanding of such events.

NIEHS
Takeda, Yukimasa
Visiting Fellow
Cell Biology - General
Retinoic acid-related orphan receptor gamma, RORgamma, coordinates the circadian regulation of energy homeostasis through the control of hepatic lipid and glucose metabolism
Defects in circadian rhythm have increasingly become a risk factor for several pathologies, including metabolic syndrome and cancer. We reported that RORgamma exhibits a strong oscillatory pattern of expression during circadian time and modulates the expression of several circadian clock genes in peripheral tissues. In this study, we reveal that RORgamma is a key transcription factor in linking the regulation of circadian rhythm to the control of lipid and glucose homeostasis. We demonstrated that energy expenditure of RORgamma-deficient (RORgamma-KO) mice as measured by oxygen consumption is strongly decreased at night time, a period at which RORgamma is most highly expressed, and not during day time. Lower respiratory exchange rate (RER) in RORgamma-KO mice suggests a preference for lipid consumption over glucose consumption in order to produce energy. Interestingly, in RORgamma-KO mice fed with a high-fat diet (HFD) the decreased energy expenditure is restored, which is likely due to the increased consumption of dietary fatty acids in RORgamma-KO mice compared to WT mice. The serum triglyceride concentration in the RORgamma-KO mice was lower than WT mice, while serum ketone bodies, largely generated by the liver, are increased in a circadian time-dependent manner. We next showed that RORgamma directly and indirectly regulates hepatic gene expression critical in lipid and glucose metabolism. In particular, RORgamma predominantly regulates circadian expression of Elovl3, elongation of very long chain fatty acids-like 3, and Avpr1a, arginine vasopressin receptor 1a, each of which regulates fatty acid biosynthesis and lipolytic activity. Also, the expression of Srebp1c and G6Pase is reduced in RORgamma-KO liver in vivo, but not in RORalpha-deficient liver, implicating that efficiency of lipogenesis and glyconeogenesis may be reduced. ChIP analysis using liver tissues showed that Elovl3 and G6pase genes are directly regulated by RORgamma through ROR-responsive elements (ROREs). In addition, microarray result shows increased gene expression of other several enzymes critical for peroxisomal and mitochondrial beta-oxidation as well as ketogenesis in RORgamma-KO liver, which supports the observation that RORgamma-KO mice more consume lipid for energy production. Our study identifies a novel physiological function of RORgamma in the coordination of the circadian regulation of energy homeostasis through hepatic lipid and glucose metabolism.

NHLBI
Barzik, Melanie
Research Fellow
Cell Biology-Cytoskeleton, Extracellular Matrix, and Structural Biology
A new member of the myosin superfamily, Myosin 18A, is highly concentrated in the dendritic spines of cerebellar Purkinje neurons: possible implications for spine morphogenesis.
Myosin 18A is a new addition to the myosin superfamily, molecular motors that convert chemical energy to mechanical energy to generate force and movement on actin. The mouse Myo18a gene encodes two splice variants, Myo18A-a and Myo18A-b, whose functions are unknown. In addition to typical motor, neck, and tail domains, Myo18A-a contains an extended N-terminus harboring an ATP-insensitive actin-binding site flanked by a region rich in Lys and Glu residues (KE region) and a PDZ domain. Myo18A-b lacks this N-terminal extension, suggesting distinct functions for these two isoforms. Myo18A is highly enriched in cerebellar Purkinje neurons, the master regulators of body movement and coordination. Purkinje neurons are rich in spines, tiny, actin-rich protrusions on the surface of dendrites that receive excitatory input from other neurons. Dynamic spines are believed to provide the morphological basis for learning, memory and cognition. However, the molecular mechanisms that modulate the number, shape and molecular composition of spines are not well understood. Myo18A has been linked to spinocerebellar ataxia, a disorder characterized by progressive loss of coordination that usually involves Purkinje neuron degeneration. Understanding the function of Myo18A in Purkinje neuron physiology may provide key insights into fundamental processes that are deregulated in neurodegenerative diseases. We found that Myo18A-a, but not Myo18A-b, localizes dramatically to dendritic spines of mouse Purkinje neurons in dissociated culture. Moreover, the N-terminal extension of Myo18A-a by itself targets to spines, arguing that it contains all domain elements required for spine localization. Function-blocking point mutations show that neither the KE region nor the PDZ domain within the Myo18A-a N-terminus determine spine targeting. Rather, interaction of the ATP-insensitive actin-binding site with actin filaments appears to control the distinctive localization of Myo18A-a. We also identified two novel Myo18A splice variants that harbor N- and C-terminal domains of unknown structure and function. Although these two splice variants also localize strongly to Purkinje neuron spines, we show that, in contrast to Myo18A-a, their N-termini alone are insufficient for spine localization. We postulate that these novel isoforms mediate interactions with distinct downstream effectors and thus present a mechanism to spatially and temporally regulate Myo18A function during neuronal development.

NIDCR
Joo, Eunyoung
Visiting Fellow
Cell Biology-Cytoskeleton, Extracellular Matrix, and Structural Biology
Myosin phosphatase coordinates the levels of contractility and acetylated microtubules to ensure normal cell migration
Effective cell migration, a process essential for normal development and physiology, requires the coordination of intracellular cytoskeletal machinery. Although much is known about how individual cytoskeletal systems contribute to cellular locomotion, how these different systems coordinate their functions to achieve physiological migration is still poorly understood. Here we show that human fibroblasts and organ explants reciprocally coordinate levels of acetylated microtubules and activity of actomyosin contraction to modulate the surface density of integrin receptors and the progression of adhesion maturation, which dictate the migration rates of fibroblasts. Experimentally reducing contraction by directly inhibiting myosin II with blebbistatin, altering the activity of myosin II by

inhibiting its kinases or indirectly by lowering cellular tension by decreasing substrate stiffness all increased the level of acetylated microtubules. On the contrary, increasing microtubule acetylation by inhibiting HDAC6, a deacetylase of microtubules, or by overexpressing a tubulin point-mutant that mimics hyperacetylation of microtubules, both decreased cellular contraction as indicated by changes of myosin light chain phosphorylation and local collagen contraction. This inverse, reciprocal interaction between acetylated microtubules and contraction was achieved by competitive myosin phosphatase interactions with either myosin light chain or HDAC6. This protein complex formation affected the activation state of either myosin II or HDAC6. Physiologically, this balance of contractility and acetylated microtubules controlled the surface density of the alpha5beta1 integrin, a receptor for fibronectin, which affected adhesion maturation into fibrillar adhesions. Hyperacetylation decreased endocytosis of the alpha5beta1 integrin and the decreased rate of migration due to hyperacetylation of microtubules was partially rescued by inhibiting the alpha5beta1 integrin. Thus, through combination of classical and novel biochemical and biophysical approaches, we have discovered that a homeostatic balance between contractility and acetylated microtubules is achieved through controlled activation and deactivation of myosin II and HDAC6, which regulates the surface density of alpha5beta1 integrin and maturation of adhesions, thereby governing the rate of cell migration.

NINDS
LEE, SEONGJU
Postdoctoral Fellow
Cell Biology-Cytoskeleton, Extracellular Matrix, and Structural Biology
The novel MIT-domain-containing protein MITD1 interacts with ESCRT-III proteins and functions in cytokinesis
The endosomal sorting complex required for transport (ESCRT) proteins are required for terminal membrane scission events in several important biological processes, including endosomal intraluminal vesicle formation, viral budding and cytokinesis. Unlike ESCRT-I and –II that are stable heteropolymeric complexes, ESCRT-III proteins are monomers in the cytoplasm and only assemble into complexes on the endosomal membrane. In particular, midbody recruitment of the ESCRT-III proteins are regulated by interactions with a series of microtubule interacting and transport (MIT)-domain-containing proteins including VPS4, spastin and AMSH. Even though many proteins are rapidly identified as MIT-domain-containing proteins, it is still not fully understood whether they also function in the ESCRT-III recruitment to the midbody. Here we identify MIT-domain containing protein 1 (MITD1) as a new interacting partner of ESCRT-III proteins. MITD1 localizes to the centrioles throughout the cell cycle and the peripheral midbody during cytokinesis. The interaction of MITD1 with all the ESCRT-III proteins are examined by yeast-two hybrid assay. Among them, only CHMP1B, CHMP2A and Ist1/CHMP8 show specific interactions with MITD1. The interactions are also confirmed by GST pull-down and co-immunoprecipitation assays. Interestingly, sequence analysis revealed that MIT domain of MITD1 is very similar to that of VPS4 proteins. The VPS4 residues identified by structure studies as required for interaction with ESCRT-III proteins are highly conserved in MITD1. Mutations in these residues of MITD1 inhibit both the interactions with CHMPs and its localization to the midbody. We also investigate a role of MITD1 in cytokinesis in HeLa cells using siRNA transfection. We found that depletion of MITD1 increases the number of multinucleated cells, reflecting impaired cytokinesis. The recruitment of CHMP1B and Ist1/CHMP8 to the midbody is also inhibited by MITD1 depletion. Currently, we are examining whether the interaction between MITD1 and ESCRT-III proteins are required for the abscission phase of cytokinesis. Our findings will provide a better understanding of the functional interactions of MIT-domain-containing proteins and ESCRT complexes as well as new insights into their roles in cell division.

NIDCD
Manor, Uri
Postdoctoral Fellow
Cell Biology-Cytoskeleton, Extracellular Matrix, and Structural Biology
Regulation of stereocilia length by myosin XVa and whirlin depends on the actin-regulatory protein Eps8.
Proper hearing and balance depends on the staircase shaped bundle of inner ear hair cell stereocilia, which are the actin-based "hairs" inside our cochlear and vestibular organs that move in response to sound and head movement. In comparison to other actin protrusions (e.g. microvilli), stereocilia are extraordinary in at least 2 ways: 1) Differential regulation - in each hair cell there are rows of stereocilia with lengths that increase in height by several micrometers, but stereocilia within the same row vary in height by no more than several nanometers. 2) Length – normal epithelial microvilli are ~500 nm long, while stereocilia are up to 120 µm long. A large number of mutations that cause deafness affect proteins involved in regulating stereocilia length. Our recent work has revealed that stereocilia are dynamic structures undergoing constant renewal and regulation via the activities of numerous myosin motor proteins and their actin-regulatory cargoes. Myosin XVa (MyoXVa) and its cargo whirlin are implicated in deafness (DFNB3 and DFNB31, respectively) and have been shown to localize at stereocilia tips and to be essential for stereocilia elongation. Given that whirlin is a scaffolding protein with no actin-regulatory activity, it remains unclear how these proteins work together to elongate stereocilia. Here we show that the actin-regulatory protein Eps8 interacts with MyoXVa and whirlin, and that mice lacking Eps8 have very short stereocilia similar to MyoXVa- and whirlin-deficient mice. We also show that Eps8 localizes to stereocilia tips in concentrations directly proportional to length, showing for the first time a relationship between the amounts of an actin-regulatory protein and stereocilia length, revealing a biochemical mechanism for differential stereocilia elongation. We show that Eps8 fails to accumulate at the tips of stereocilia in the absence of MyoXVa, that overexpression of MyoXVa results in both elongation of stereocilia and increased accumulation of Eps8 at stereocilia tips, and that the exogenous expression of MyoXVa in MyoXVa-deficient hair cells rescues Eps8 tip localization. Our data demonstrates that MyoXVa, whirlin, and Eps8 are integral components of a stereocilia tip complex, where Eps8 is a central actin-regulatory element transported by MyoXVa to stereocilia tips for elongation of the stereocilia actin core. This work provides insight towards DFNB3 and DFNB31 pathologies, and identifies EPS8 as a candidate deafness gene.

NIAID
Petrie Aronin, Caren
Postdoctoral Fellow
Cell Biology-Cytoskeleton, Extracellular Matrix, and Structural Biology
Combining 4D Imaging with Microfluidics to Study Innate Immune Cell Migration In Vitro
Unlike fibroblastic cells that utilize enzymatic tools to degrade basement membrane structures, innate immune cells must efficiently travel to distal sites of tissue infection and home to the lymphatics without significant tissue re-organization. Thus, because most interstitial spaces are tightly packed with cells and matrix fibers, it is plausible that leukocyte migration utilizes native features of the µ-environment to optimally translocate to sites of importance. Complicated in vivo µ-environments can limit manipulation of extracellular input parameters. Therefore, addressing questions of leukocyte migration have long been limited to 2D systems, including the pipette tip, under-agarose, and Boyden chambers assays. With the advent of µ-fluidic technology, definable 3D environments are now technically possible and warrant transition for many biological systems from 2D to 3D platforms. Here we describe a novel µ-fluidic platform with multi-modal capabilities. Specifically, this device can a) create definable chemokine gradient inputs, b) control fiber orientation ranging from random to aligned, and c) collect real-time 4D images of both cellular migration (CD11c-YFP mature dendritic cells [DCs])

and resultant fiber deformations (AF647-collagen) in response to soluble CCL19 chemokine gradients. Using syringe pumps to control flow, we can establish either stable or temporally changing gradients of soluble CCL19 across the cell-matrix channel for up to 4 hrs of visualization. Most interestingly, long-term stable gradients of CCL19 significantly reduce directed migration of mature DCs. These results are in stark contrast to many 2D studies which report directed migration in stable gradients. Our observations suggest that temporal changes in gradient profile may play an important role for cells navigating through 3D spatial volumes as compared with confined 2D infinite planes. By reducing μ-channel width, the resulting collagen network transitions fiber orientation from random to >80% aligned. High-resolution 4D image capture of cellular migration under similar chemokine gradients but differing matrix alignment, show reduced migration speeds in aligned fiber environments suggesting orthogonal forces exerted by forward protrusions may not be as efficiently applied in parallel networks. This robust platform showcases the manipulation of various extracellular parameters to understand μ-environmental conditions which influence leukocyte directed migration.

NCI-CCR
Daniel, Jeremy
Postdoctoral Fellow
Cell Cycle-General, Regulators and Checkpoints, Apoptotic Mechanisms
Loss of DNA damage-induced ATM kinase activity leads to embryonic lethality in mice
The ATM kinase becomes activated at sites of DNA double-strand breaks (DSBs) and is a key signaling kinase in the DNA damage response, phosphorylating numerous substrates on damaged chromatin to regulate cell cycle checkpoints, radiation resistance, meiotic and lymphocyte recombination events, and to protect against oncogenic translocations. In addition to signaling DNA damage, ATM may also play a more direct role in promoting DSB repair. The genomic instability syndrome ataxia telangiectasia (A-T) is caused by mutations in the Atm gene, most of which lead to undetectable ATM protein and therefore loss of ATM kinase activity. However, a few classic A-T patients with deficient ATM kinase activity continue to express near normal ATM protein levels. To test whether in vivo ATM functions are mediated solely by its kinase activity, we generated two mouse models containing single point mutations in the phosphatidylinositol-3-kinase-(PI3K)-related kinase domain of ATM; one based on structural modeling within the hypothesized active site and the other based on a missense mutation from a patient with classic A-T. Here we show that the D2899A and Q2740P point mutations lead to loss of in vitro kinase activity without affecting protein stability. Surprisingly, each single mutation in Atm causes embryonic lethality in mice. The D2899A mutation impairs development of the mouse before embryonic day 9.5. We show that early embryonic lethality associated with loss of ATM kinase activity cannot be rescued by disruption of Ku80 or 53bp1. To circumvent the observed embryonic lethality, we have used a conditional deletion approach to express the D2899A mutant from of ATM as the sole ATM species in B lymphocytes. ATM D2899A mutant B cells display loss of in vivo kinase activity, decreased fitness, and increased sensitivity to PARP inhibition compared to control cells expressing no ATM protein. Further studies using these mouse models may provide insight into how ATM kinase activity regulates dynamics of repair proteins at damaged chromatin. Our observations indicate that inhibition of ATM kinase activity in vivo is not phenotypically equivalent to loss of ATM protein. These results have important implications for the usage of ATM inhibitors for radiosensitization of cancer patients as well as in experimental systems.

NCI-CCR
McCollum, Andrea
Postdoctoral Fellow
Cell Cycle-General, Regulators and Checkpoints, Apoptotic Mechanisms

A novel function of WW domain binding protein 2 (WBP2) in regulating cytoskeletal function and cellular division through binding to co-chaperone BAG3

The members of the BAG protein family are characterized by a C-terminal BAG domain that directly binds to the ATPase domain of Hsp70/Hsc70. This binding prevents the release of Hsp70/Hsc70 client proteins that would otherwise be directed to the proteasome for degradation. BAG3 also contains an N-terminal tryptophan domain (WW), deletion of which we have shown to cause an enlarged, abnormal cellular morphology, degenerate F-actin organization, a significantly longer doubling time (p<0.05, n=4), and a three-fold increase in the number of polynucleated cells relative to both empty vector and full length-BAG3 (FL-BAG3; p<0.001, n=3). Additionally, we have shown the BAG3 WW domain binds to F-actin and co-localizes with actin filaments during G2 and M phases of the cell cycle. However, the mechanism by which the WW domain interaction with F-actin is regulated during the cell cycle has not been identified. Therefore, we hypothesized cell cycle-dependent binding of a novel interacting protein to the BAG3 WW domain regulates BAG3-F-actin binding. We performed a yeast two-hybrid assay and identified WBP2, a WW domain binding protein with no known function, as a novel BAG3 interactor. WBP2 was pulled down with GST-WW, but not GST control, indicating a specific interaction between WBP2 and the BAG3 WW domain. Immunoprecipitation using a series of V5-tagged domain deletion BAG3 mutants demonstrated that all mutants, except those with a deleted WW domain, bound to WBP2. BAG3 and WBP2 co-immunoprecipitated in G1 and S phases, but not G2 or M, indicating that the binding of BAG3 to WBP2 is cell cycle-specific. Silencing of WBP2 results in BAG3 co-immunoprecipitation with F-actin indiscriminately across all cell cycle phases, indicating that association with WBP2 prevents BAG3 binding to F-actin during specific cell cycle phases. We are the first to identify a function for the novel BAG3-interacting protein WBP2, which our data show regulates the BAG3 WW-domain association with the actin cytoskeleton in a cell cycle specific manner. Taken together, our findings show a novel mechanism through which regulation of BAG3 location drives proper cell division. Because actin dysfunction has long been proposed to contribute to chromosomal instability and tumor formation, the WW-domain of BAG3 is a potential therapeutic target to reduce abnormal cell division in cancer.

NICHD
MO, MIN
Visiting Fellow
Cell Cycle-General, Regulators and Checkpoints, Apoptotic Mechanisms

Distinct roles for Hec1 phosphorylation by Aurora B and Mps1 in controlling chromosome segregation

Defective chromosome segregation must be avoided as it produces daughter cells with aneuploidy (abnormal chromosome number), a genetic alteration found in many cancers. A microtubule (MT)-based structure called the spindle mediates chromosome segregation, through binding of spindle MTs to kinetochores (KTs) on the centromeres of each sister chromatid. A regulatory pathway called the Spindle Assembly Checkpoint (SAC) monitors the attachment of spindle MTs to KTs. The SAC delays the onset of anaphase until all KTs are correctly attached and aligned on the metaphase plate. One crucial KT protein is Hec1 (highly expressed in cancer 1), part of a complex that couples MT dynamics to chromosome movements. The Aurora-B kinase phosphorylates Hec1 as part of an error-correction mechanism for mis-attached KTs. Particularly, Aurora-B phosphorylation of Hec1's unstructured N-terminal domain on at least five sites alters the charge of this domain and allows MT release. We found that Hec1 is also phosphorylated by the Mps1 (monopolar spindle 1) kinase, a SAC protein. Using antibodies specific to a peptide from Hec1's N-terminal domain with two phosphorylated serine residues, we observed staining of kinetochores under conditions where the SAC was active. Surprisingly, this phosphorylation was sensitive to Mps1 inhibitors but not to Aurora-B inhibitors. To investigate further, we made a phosphomimetic mutant with both serine residues changed to aspartic acids (DD mutant) and a non-

phosphorylatable mutant with both changed to alanines (AA mutant). Contrary to models wherein phosphorylation of at least five residues is needed to abolish MT-KT interaction, we found that MT-KT association was disrupted in cells expressing the DD mutant, arresting them in mitosis. In contrast, cells expressing the AA mutant had a reduced mitotic index, suggesting either that they were unable to enter mitosis or that the duration of mitosis was greatly shortened. In summary, our findings show that phosphorylation of Hec1 by Mps1 plays a critical role in its regulation that is distinct from the earlier phenotypes defined for phosphorylation by Aurora-B. These findings suggest a complex and multi-dimensional pattern of Hec1 regulation to assure the fidelity of chromosome segregation and prevent aneuploidy. Currently, we are performing time-lapse microscopy on mitotic cells expressing both mutants to define the role of Hec1 phosphorylation by Mps1 and especially its relationship with the SAC.

NCI-CCR
Soto Pantoja, David
Postdoctoral Fellow
Cell Cycle-General, Regulators and Checkpoints, Apoptotic Mechanisms
The Role of autophagy in the radioprotection of normal tissue and delayed tumor growth by blockade of CD47 signaling.
Over half of all cancer patients will require irradiation (IR) therapy for the clinical management of their disease however a major problem with this therapy is damage to normal tissue limiting treatment dosages to achieve radiocurability. CD47 is an integral membrane protein with an N-terminal immunoglobulin domain and a C-terminal multiple membrane-spanning region. CD47 is a receptor for the secreted protein thrombospondin-1 (TSP-1). Binding of TSP-1 to CD47 on vascular cells inhibits nitric oxide (NO) signaling. Previously it was demonstrated that antagonists of this pathway improve tissue survival of ischemic injuries, we now show that this protective activity extends to radiation injuries. Soft tissues and bone marrow in mice lacking either TSP1 or CD47 are profoundly resistant to doses of ionizing radiation used for radiotherapy of cancer. Lack of CD47 both enhances cell survival and preserves proliferative capacity post-irradiation. However, in a syngeneic model irradiated tumors are not protected by the lack of CD47 and instead show markedly greater regrowth delays reducing tumor volume by 40% when compared to tumors of irradiated WT animals. Treatment with morpholinos to block CD47 confers a similar radioprotection and tumor regrowth delays, moreover blockade of CD47 also increased survival of mice exposed to total body irradiation by 80% when compared to 50% in WT mice. Our data indicates that the observed effects may be mediated induction of autophagy genes. Autophagy is a mechanism activated in eukaryotic cells in response to metabolic and other types of stress allowing cell survival. Our experiments demonstrate that irradiated T cells lacking CD47 have significant increases in Beclin-1 (1.2±0.27-WT vs. 4.9 ± 0.89-CD47-/-) and ATG-5 (0.33 ± 0.11-WT vs. 4.2 ± 1.14-CD47-/-). We also observe p62/sequestosome protein degradation and increased autophagosome formation measured by immunofluorescent LC3 antibody relative to irradiated wild type T cells. Activation of the autophagy program is a known response of tumor cells to radiation, suppression of CD47 in B16 melanoma cells did not further induce the expression of these genes after radiation. There are no reports that the TSP-1/CD47 axis is involved in regulating autophagy. The selective activation of autophagy is a novel paradigm that may explain the radioprotenction and tumor ablation observed in our in vivo animal models.

NCI-CCR
Adams, Kristie
Postdoctoral Fellow
Chemistry

Structural studies of antiproliferative factor glycopeptide analogs: Synergistic peptide and sugar conformational effects
Glycosylated proteins are ubiquitous both within and on the extracellular surface of eukaryotic cells. Specific glycosylation patterns are critical for the proper function of a host of cellular processes. Aberrant glycosylation is implicated in a wide variety of human diseases, including cancer. Glycosylation affects overall three-dimensional structure and conformation of the protein backbone, and thus affects proper protein folding and assumption of active tertiary structure. Study of glycosylated peptides and proteins is hampered by the difficulty of obtaining pure material; thus, structural information of glycopeptides is severely lacking. Our lab synthesizes and analyzes glycopeptides derived from fragments of cell surface proteins. Significant efforts are dedicated to a small naturally occurring glycopeptide from the urine of interstitial cystitis patients called antiproliferative factor or APF (viz. Neu5Aca2-3Galß1-3GalNAca-O-TVPAAVVVA). We have synthesized more than 65 analogues of asialo-APF (as-APF), the equally potent desialylated version of the glycopeptide. We have studied the structures of several more intriguing analogues by NMR spectroscopy and molecular modeling. as-APF is the first glycopeptide studied containing an N-terminal glycosylation site, and we have found that the disaccharide controls the local conformation of the peptide around the glycosylated residue. Glycosylation also affects aspects of the peptide conformation, and small changes to the amino acids in the N-terminal domain also have complementary effects on the disposition of the disaccharide. The results from extensive NMR spectroscopic data coupled with molecular dynamics performed with the newly derived CHARMM36 force field optimized for carbohydrates, will be presented and discussed.

NCI-CCR
Bahta, Medhanit
Postdoctoral Fellow
Chemistry
Development of Nanomolar Affinity Inhibitors of the Yersinia Pestis Protein-Tyrosine Phosphatase (YopH)
The protein-tyrosine phosphatase (PTP), YopH serves as an essential virulence factor for Yersinia pestis (Y. pestis), the causative pathogen of plague and a potential bioterrorism agent. In spite of the significant progress been made in inhibitor development against certain other phosphatases, YopH represents an important yet much less developed therapeutic target. Our current study reports the first K subscript-M optimization of a library of nitrophenylphosphate containing substrates for the purpose of generating a PTP inhibitor lead. A high affinity substrate identified by this method (K subscript-M = 80 uM versus K subscript-M = 600 uM for parent p-nitrophenylphosphate) was converted from a substrate into an inhibitor by replacement of its phosphate group with the non-hydrolyzable phosphoryl mimetic, difluoromethylphosphonic acid and by attachment of an aminooxy handle to provide a site for further structural optimization by oxime-ligation. A cocrystal structure of this aminooxy platform in complex with YopH allowed unambiguous assignment of its binding orientation and the identification of a conserved water molecule proximal to the ligand aminooxy group that was subsequently employed for the design of furanyl-based oxime derivatives. By this process a non-promiscuous high affinity (IC subscript-50 = 190 nM) inhibitor was developed that exhibited good YopH selectivity relative to a panel of phosphatases. The inhibitor showed significant inhibition of intracellular Y. pestis replication at a non-cytotoxic concentration. The current work presents general approaches to PTP inhibitor development that may afford significant utility beyond its immediate target.

NIAID
Marriner, Gwendolyn
Postdoctoral Fellow
Chemistry

Development of PET Radiotracers based on Small Molecule Antitubercular Drugs
Tuberculosis (TB) causes nearly 2 million deaths annually, and 2 billion people have either active or latent infection. New drugs are desperately needed to limit the spread of drug resistant strains. The ability of drugs to penetrate TB lesions in the lung may be a predictor of efficacy of new TB treatments, but traditional biodistribution studies are labor-intensive and require euthanizing study animals. Using 18F to radiolabel small molecule antitubercular drugs would allow Positron Emission Tomography (PET) imaging to analyze biodistribution of both novel and existing drugs in real-time to assess which compounds have the highest probability of becoming effective TB treatments. This would allow more rapid selection of potential new drugs from a large pool of candidates. Additionally, PET imaging offers a non-invasive way to monitor disease state in TB patients undergoing treatment. Our group has pioneered the use of [18F]-fluorodeoxyglucose (FDG) to monitor treatment response during human chemotherapy. A limitation of this approach, however, is that FDG concentrates in areas of high glucose metabolism, which can indicate inflammation or other secondary markers of infection. Imaging agents that respond to bacterial load rather than secondary markers of infection would provide powerful tools to evaluate whether chemotherapy is effective. We have synthesized and evaluated analogs of two drugs in clinical trials for treatment of TB, an [18F]-linezolid analog and a [18F]-PA-824 analog in vivo using TB-infected rabbits. The [18F]-linezolid analog showed biodistribution differing from that of the original compound, suggesting that this analog is perhaps distributed differently than the parent linezolid. A second-generation synthesis will label the linezolid at its native position in a method applicable to other analogs in clinical development. The [18F]-PA-824 showed a 2-3-fold signal increase in a lung lesion compared to healthy tissue but also high levels of [18F]-defluorination. A second generation approach to PA-824 analogs incorporating a more robust radiolabel is ongoing. Additionally, we are currently pursuing routes to other potential imaging agents used to treat TB. This research should introduce new radiotracers for imaging currently infected patients to monitor whether chemotherapy is proceeding successfully as well as decrease development time for new TB treatments.

NIDDK
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Chemistry
Modification of pyrimidine nucleotides with 4-alkoxyamino and d-esters of terminal phosphate as selective agonist of the P2Y4 receptor
Abstract The P2Y4 receptor is a G protein-coupled receptor (GPCR), activated by UTP and dinucleoside tetraphosphates, which is widely distributed in epithelial cells, smooth muscle, and other tissues. We modified the phosphate and uracil moieties in analogues of pyrimidine nucleoside 5'-triphosphates and 5'-phosphoesters, which were evaluated in stimulation of phospholipase C in stably transfected 1321N1 astrocytoma cells. The 4 position of cytidine was appended with alkyloxy groups, which enhanced P2Y4 receptor potency. Hydroxyl groups on a terminal d-glucose phosphoester of uridine 5'-tetraphosphate were either inverted or substituted with hydrogen or fluorine to probe the effect of H-bonding in molecular recognition. N4-(Benzyloxy)-CTP (MRS2976) and Up4-[1]3'-deoxy-3'-fluoroglucose (MRS2927) were selective for the P2Y4 receptor with EC50 values of 97 and 62 nM, respectively. Thus, the potency, selectivity, and stability of extended uridine tetraphosphate derivatives may be modulated by distal structural changes, and we have identified the first reported P2Y4 receptor agonists.

NCI-CCR
NANDURDIKAR, RAHUL
Visiting Fellow
Chemistry

Nitric Oxide-Releasing Drugs in Cancer Therapy: Mechanisms of Action and Lead Optimization
Nitric oxide (NO) is a multifaceted bioregulatory molecule with potential applications in treating several diseases, including cancer. Diazeniumdiolate prodrugs, such as JS-K are a growing class of promising NO-based therapeutics. JS-K is currently in late pre-clinical development as an anti-cancer drug candidate. JS-K was designed to be activated by glutathione (GSH) to release NO, and this reaction is catalyzed by glutathione S-transferase (GST), a detoxifying enzyme frequently over-expressed in cancer cells. We have shown that JS-K is a potent cytotoxic agent against human non-small cell lung cancer (NSCLC) cell lines both in vitro and as xenografts in mice. We investigated the mechanism(s) of anticancer activity of JS-K against NSCLC cells. The drug is most effective in cells characterized by high endogenous levels of reactive oxygen species (ROS) and low levels of antioxidant defense/DNA repair mechanisms. Treatment of NSCLC cells with JS-K generated oxidative/nitrosative stress in cells with high basal levels of ROS. This, in combination with the arylating properties of the drug, was reflected in glutathione depletion and alteration in cellular redox potential, mitochondrial membrane permeabilization and cytochrome c release. Inactivation of MnSOD by nitration was associated with increased superoxide and significant DNA damage. Apoptosis followed these events. Taken together, our data suggest that NO–releasing prodrugs may have application as a personalized therapy for cancers characterized by high levels of ROS. To improve on this lead, we are currently seeking to design JS-K analogues that are activated to release NO only when catalyzed by GST isoforms that are prevalent in a given tumor tissue. The aryl group "caging" the NO-generating diazeniumdiolate moiety is a key structural feature that controls this reaction, so we are studying the effects of changing substitution patterns in the aromatic ring for a number of piperazine and homopiperazine analogues of JS-K with the aim of slowing the rate of their uncatalyzed reaction with GSH and subsequently improving their anticancer properties. This and previous structure-activity relationship studies provide insight into a common structural motif for anticancer activity on the part of drug candidates in the arylated diazeniumdiolate series.

NCI-CCR
Abuhatzira, Liron
Visiting Fellow
Chromatin and Chromosomes
The Chromatin Binding Protein HMGN1 Regulates The Expression Of MeCP2 And Affects The Behavior Of Mice.
High Mobility Group N1 protein (HMGN1), a nucleosomal binding protein that affects the structure and function of chromatin, is encoded by a gene located on chromosome 21, in a region associated with Down syndrome. Misexpression of HMGN1 affects the cellular transcription profile; however, the biological function of this protein and its possible role in the etiology of Down syndrome are not known. To gain insights into these questions we used ChIP sequence analysis to test for genes whose expression could be directly affected by HMGN1, in human and mouse brains. Bioinformatics analysis revealed that HMGN1 localizes to chromatin regions containing genes associated with mental retardation and neurological disorders, including the promoter of the DNA-binding protein Methyl CpG binding Protein 2 (MeCP2). Mutations or duplication of MeCP2 are known to result in several neuro-developmental disorders including Rett syndrome, mental retardation, autism spectrum disorders and MeCP2 duplication syndrome. In addition, expression analysis to brain tissues from Down syndrome patients and their age matched controls show that MeCP2 is down-regulated in Down syndrome cases. Quantitative PCR and Western analyses using brain tissues from mice that over-express HMGN1 and from Hmgn1-/- mice relative to control mice, revealed that the up-regulation of HMGN1 is associated with down-regulation of MeCP2 expression while knock-out of the Hmgn1 gene leads to up-regulation of MeCP2, suggesting that HMGN1 is a negative regulator of MeCP2 expression. Formaldehyde Assisted Isolation of Regulatory Elements (FAIRE) and ChIP analyses revealed that altered expression of HMGN1

is accompanied by changes in chromatin modifications and structure around the MeCP2 promoter. Behavior analyses using Open Field test, Elevated Plus Maze, Reciprocal Social Interaction, and Automated Sociability test, revealed that changes in HMGN1 expression lead to abnormalities in activity levels, reaction to fear, sociability, and novel object preferences. Our results indicate that HMGN1 directly modulates the expression of MeCP2 and raise the possibility that misexpression of HMGN1 may lead to neurological phenotypes including some of those observed in Down syndrome patients.

NCI-CCR
Baranello, Laura
Postdoctoral Fellow
Chromatin and Chromosomes
Genome-wide mapping of Topoisomerase 1 activity reveals its biphasic role in gene transcription
Topoisomerase 1 (Top 1) regulates the topological state of DNA and enables essential processes such as DNA replication, transcription and recombination by transient cleavage of DNA. Although one of the main roles of Top 1 has been presumed to be the relaxation of torsional stress generated during transcription; until now it has not been directly shown. To determine the precise function of Top 1, we developed a method to map Top 1 cleavage sites at the genome-wide scale. Unlike the common ChIP-based assays, this method reveals sites of Top 1 functional activity allowing us to investigate Top 1 involvement in genome function. We found that Top 1 is preferentially active at intragenic regions and is mainly localized near the 5’ and 3’ ends of genes. At the gene level, the Top 1 distribution strictly correlates with RNAP II localization suggesting an association of Top 1 with the transcription machinery. Intriguingly, two separate populations of Top 1 cleavage sites were found at transcribed genes. The first is distributed along the bodies of genes and is involved in relaxation of transcription-generated supercoils, and the second is localized near transcription start sites. Upon Top 1 knockdown, the second population of enzyme is virtually eliminated causing an accumulation of RNAP II at transcription start sites suggesting an involvement of Top 1 in the regulation of promoter escape. The first population of enzyme did not change its activity which suggests that Top 1 is highly essential for elongation of transcription but can be compensated at 5' ends of genes. By using campthotecin as in vivo modulator of Top 1 processivity, we confirmed these observations. The drug causes RNAP II to accumulate downstream of the transcription start sites, especially in Top 1 knockdown cells. This result indicates that high torsional stress impedes the movement of RNAP II in the genes’ bodies and affects the reloading of promoters. Our study provides new insights into Top 1’s role during transcription and links its activity to the regulation of distinct transcriptional phases in vivo. We show for the first time a biphasic role for Top 1 not only as an elongation factor essential for the progression of the transcription machinery through genes' bodies, but as a general transcription factor involved in the initial phases of transcription.

NCI-CCR
Burgess, Rebecca
Postdoctoral Fellow
Chromatin and Chromosomes
Characterization of a local heterochromatin-induced DNA damage response
While chromatin modifications and remodeling have long been appreciated as vital components of many processes requiring access to DNA at the sequence level, such as DNA replication and transcription, the role of chromatin in processes of DNA repair and DNA damage response (DDR) is just beginning to be understood. Large-scale chromatin remodeling events may facilitate the recognition and repair of damage. While the mechanisms remain elusive, it has been shown that targets of the ATM kinase are required for repair in the context of heterochromatin. In addition to large-scale events,

histone modifications may modulate DDR by recruiting factors and directing downstream repair events. It has also been suggested that DDR could be activated directly by alterations in chromatin structure. To investigate the role of chromatin structure in DDR, we are using a protein-chromatin tethering system to create chromatin domains adjacent to an inducible double strand break site in human cells. When we produce detectably condensed chromatin domains by tethering constitutive heterochromatin factors (HP1 variants, Su(var)3-9, and histone H1 variants),we see recruitment of DDR proteins such as gamma-H2AX, as well as checkpoint and repair factors. Conversely, we do not observe DDR activation upon tethering of chromatin opening factors such as the SWI/SNF subunit BRG1 or VP16. We confirmed that the DDR activation we see upon tethering heterochromatin factors is indeed linked to the condensed state of the chromatin and not due to DNA damage, using TUNEL and ligation-mediated PCR assays. We identified the ATR as the key kinase for the DDR activation. The ATR requirement for this heterochromatin-based DDR bears a resemblance to oncogene-induced senescence, in which an ATR-dependent DDR accompanies the appearance of senescence-associated heterochromatin foci. While we observe robust recruitment of DDR factors to the heterochromatin domains, this DDR appears to be local in nature as we do not observe the typical markers for cell-wide DNA damage signaling, such as induction of gamma-H2AX, p53 phosphorylation, or checkpoint arrest. Other methods of unscheduled chromatin condensation (e.g. treatment with Calyculin A) can also activate the DDR. We are currently continuing our investigations into DDR activation in response to unscheduled chromatin compaction and its role in global genome surveillance, as well as potential impacts on oncogene-induced senescence.

NIAMS
Mousavi, Kambiz
Visiting Fellow
Chromatin and Chromosomes
Role of Polycomb Protein Ezh1 in RNA Polymerase II-mediated Transcription
Chromatin modifiers such as Polycomb group (PcG) proteins are known for their ability to modulate gene expression in health and disease states. A mammalian PcG protein, Enhancer of Zeste homolog 2 (Ezh2), triggers transcriptional repression of developmental genes by catalyzing the addition of methyl groups onto lysine-27 of histone H3. This action facilitates the binding of other PcG proteins to chromatin for purposes of gene silencing. Upon maturation of various mammalian tissues and organs, the expression of Ezh2 is diminished and its levels become negligible. This observation prompted us to explore for suitable substitutes with similar enzymatic activity in fully matured cells. Here, we define a role for an evolutionary related PcG protein, Ezh1, in a model of cellular differentiation and in this case myogenesis. By Chromatin ImmunoPrecipitation and deep Sequencing (ChIP-Seq), we describe Ezh1 occupancy profile relative to known genomic landmarks. Unpredictably and in contrast to Ezh2, Ezh1 occupancy profile overlaps predominately with active chromatin modifications and transcriptional machinery, RNA polymerase II (PolII). To define its role within transcribed genomic regions, Ezh1 was depleted by RNA interference (Ezh1i), resulting in significant reduction in its occupancy genome-wide. In these experiments, Ezh1i causes PolII deficiency within gene bodies, suggesting its involvement in promoting PolII-mediated transcription. Specifically, Ezh1i leads to depletion of elongating form of PolII genome-wide and consequently reduction of majority of mRNA species in skeletal muscle cells. Furthermore, Ezh1i causes delayed activation of gene expression during myogenic differentiation. In inverse experiments, cells over-expressing Ezh1, and not its catalytically mutant isoform, initiate premature activation of developmental genes during myogenic differentiation. In fact, re-introduction of Ezh1 rescues global PolII deficiency observed in Ezh1-depeleted cells. The results obtained from these experiments suggest that the catalysis conferred by Ezh1 primes or accelerates PolII-mediated transcription. Collectively, these findings reveal an unanticipated role for a PcG complex in promoting mRNA transcription and can be instrumental toward molecular therapies in myopathies.

NICHD
Cluzeau, Celine
Visiting Fellow
Clinical and Translational Research
Abnormal gene expression profiles in Npc1 mutant mouse livers
Niemann-Pick type C (NPC) is a lethal, autosomal recessive neurodegenerative disorder, resulting from mutations in NPC1 and NPC2 genes, with no approved therapy. Cholesterol and sphingolipids are trapped in endosomes/lysosomes, which results in liver disease and progressive neurodegeneration. The diagnosis is usually difficult and delayed, as most of the clinical signs fit many differential diagnoses. Despite intensive work, the mechanisms leading to both brain and liver dysfunctions are poorly understood. In order to identify functional pathways responsible for the NPC pathology, we performed a microarray analysis using the murine Npc1-/- model, which closely recapitulates the human disorder. We collected liver tissue from mutant and control littermates (N=4), at different pre- (1-, 3- and 5-week old) or post-symptomatic stages (7-, 9- and 11-week old). Using this unique approach, we identified approximately 200 modified pathways. These included altered gene expression in pathways corresponding to metabolic processes, immune response and developmental signaling. In a list of 155 differentially expressed genes (DEG) common to all time points, we surprisingly identified 10 downregulated genes from the cytochrome P450 family, which we validated by qPCR. P450 cytochromes are involved in the metabolism of drugs and endogenous compounds, such as arachidonic acid (AA). AA metabolites include the pro-inflammatory molecules prostaglandins (PG) and leukotrienes (LT). We then hypothesized that the inflammation seen in NPC could result from a dysregulation in AA metabolism. We are testing if there is a deregulation of PG and LT levels in NPC patients' cerebral spinal fluid. The early modification of cytochrome P450 system would be an appealing target for treatment, as approved anti-inflammatory drugs targeting this pathway are available. This is also a significant pharmacogenetic finding since impaired P450 activity may result in altered drug metabolism by NPC patients and thus require alteration in medication dosing. We have proven a marked reduction in drug metabolism in the model mice, and will characterize P450 system activity in human NPC and control livers. This microarray study also identified 103 DEG encoding secreted proteins, including 12 with a modified expression at all ages. This group of proteins may be particularly useful as biomarkers to facilitate diagnosis, or used as outcome measures in screening of drugs with potential therapeutic efficacy.

NHGRI
Flyn, Elizabeth
Postdoctoral Fellow
Clinical and Translational Research
Comparative Genomic Hybridization (CGH) using a high-density oligo array for discovering deletions in all the Fanconi anemia genes
Fanconi Anemia (FA) is an autosomal recessive disorder that is highly associated with bone marrow failure, congenital abnormalities, chromosomal instability, and increased risk of cancer. Mutations in 14 genes, including FANC-A, B, C, D1, D2, E, F, G, I, J, L, M, N, and most recently P, have been shown causative for Fanconi Anemia. These genes have a common functional role in DNA repair pathways. In a collaborative effort with the International Fanconi Anemia Registry (IFAR) to understand genomic and phenotypic correlations, we have used comparative genomic hybridization (CGH) using high-density oligo arrays to detect deletions in Fanconi Anemia patients. CGH allows for high throughput and efficient identification of genomic deletions and duplications at a high resolution. Unlike the current methods, the analysis is not limited only to the exonic regions, and therefore can identify the precise ends of a deletion. The high-density arrays accommodate the entire genomic regions of all the FA genes (and

many other genes of interest as well), and thus can screen for deletions in all the FA genes at once. Thus far, CGH analysis has been collected on DNA from 72 FA patients, which included several controls with known deletions. 50 DNAs assigned to FANCA gene mutation were analyzed and CGH arrays identified deletions in 27 samples. Similarly deletions were identified in one each of the two DNAs tested from the FA-B, FA-C and FA-G groups. We were able to identify over-lapping deletions in a compound heterozygous sample as well as other homozygous deletions, and the precise breakpoints of both the deletions could be inferred from the data. We found a deletion in the FANCA gene in a sample with no prior assigned complementation group. One benefit of the high resolution afforded by the CGH array is the ability to map exact genomic breakpoints. We are currently in the process of verifying the CGH results by amplification across the breakpoint followed DNA sequencing. We are particularly interested in analyzing samples with a shared common deletion interval. The CGH arrays also have translation applications as a complementary tool for a quick and efficient molecular diagnosis of FA. In addition, deletions in FANCC and another in FANCD1 gene have been reported in two pancreatic cancer cell lines, and a comprehensive screening approach would be helpful in scanning for FA gene deletions in pancreatic and other cancers.

CC

Gacchina, Carmen
Postdoctoral Fellow
Clinical and Translational Research

Low Temperature Sensitive Liposome Mediated Delivery of Thrombolytic Agents

Thromboembolic disease (e.g. myocardial infarction, stroke, deep vein thrombosis) causes significant morbidity and mortality worldwide. Thrombolytics have been shown to effectively dissolve thrombi and recanalize occluded blood vessels. However, their effectiveness is limited clinically by lack of specific delivery, which necessitates large therapeutic doses, resulting in systemic toxicity. Temperature-sensitive liposomes preferentially release their contents upon reaching mild hyperthermia. Together with a hyperthermia applicator, such liposomes have the potential for local, sustained delivery of thrombolytic agents at the site of a heated thrombus. The objectives of this study were to formulate liposomes encapsulating thrombolytics, characterize release, and ensure thrombolytic activity following hyperthermia. Three liposome formulations were investigated. The enzymatic thrombolytics staphylokinase (SAK), urokinase (UK), or tissue plasminogen activator (tPA) were loaded into each formulation. To characterize time and temperature dependence of a high MW cargo from each formulation, fluorescein-conjugated dextrans (70kDa) were loaded (25mg/ml) and release was quantified with spectrophotometric methods. Thrombolytic leakage (37 degrees C) and activated release with heating at the temperatures that exhibited maximal release rate (38-44 degrees C) were quantified via chromogenic enzymatic activity assays to determine the thrombolytic-liposome formulation with the highest release/leakage ratio. Clot lysis was evaluated by measuring clot mass. The SAK-loaded low temperature sensitive liposomes (LTSLs) yielded significant advantages over other thrombolytic-liposome pairs. Release of dextrans loaded in LTSLs was observed to be 11.5+/-1.5%, 79.7+/-1.6%, and 94+/-4% after 15 min in plasma at 37, 39, and 41.3 degrees C, respectively. SAK LTSL had the highest release/leakage ratio of 8.3+/-0.6. SAK LTSL also demonstrated the greatest whole blood clot lysis with hyperthermia (84+/-8% and 45+/-8%, at 42 and 37 degrees C, respectively). p 0.05; n=3. The SAK LTSL is a temperature-sensitive thrombolytic-liposome formulation that can potentially deliver thrombolytics selectively when combined with local hyperthermia, thus providing sufficient thrombolysis while minimizing systemic side effects. Feasibility studies combine this liposome formulation with magnetic resonance-guided high intensity focused ultrasound for image-guided focal heating and clot lysis.

NCI-CCR
Kerkar, Sid
Clinical Fellow
Clinical and Translational Research
IL-12 indirectly enhances murine CD8+ T-cell immunity by reprogramming in-situ myeloid-derived cells to cross-present antigen
Myeloid-derived cells comprising the tumor stroma represent a heterogeneous population of cells critical to the structure, function and growth of established cancers. We have recently found that engineering tumor-specific pmel-1 CD8+ T cells to secrete IL-12 (P-IL-12) can lead to striking improvements in T-cell activity against B16 melanomas. To understand how supra-physiological levels of IL-12 within tumors allowed pmel-1 CD8+ T cells to break tolerance, we performed whole transcriptome analysis of tumor samples and found an effector T cell and interferon-gamma centered inflammatory gene signature from tumors treated with P-IL-12 cells compared to non-transduced pmel-1 CD8+ T cells. Surprisingly, we found that P-IL-12 cells that lacked the ability to receive signals from IL-12 (Il12rb2-/-) and indeed T cells that lacked the ability to produce IFNy (Ifny-/-), retained all of their ability to trigger tumor destruction. However, anti-tumor efficacy was abrogated when the cells in host mice lacked IL-12 receptors (Il12rb2-/-), IFN-y receptors (Ifnyr-/-) or the ability to produce IFN-y (Ifny-/-). Interestingly, tumor treatments remained intact in mice devoid of T, B, and NK cells (Rag1-/- + NK1.1 depletion), indicating that IL-12 indirectly enhanced the function of adoptively transferred T cells by sensitizing the innate immune arm in an interferon-gamma dependent manner. We confirmed this hypothesis by transferring P-IL-12 cells (H-2b restricted) into mice deficient in class I (B2m-/-) and observed an impairment in anti-tumor activity. However, when P-IL-12 cells were transferred into DBA/F1 H-2b/d mice bearing H-2d Cloudman S91 melanomas, anti-tumor responses remained intact, indicating the functional importance of cross-presentation in vivo. Furthermore, we observed the elimination of CD11b+ F4/80+ macrophages, CD11b+ ClassII+ CD11c+ dendritic cells, and CD11b+ Ly6C+ Ly6G- but not CD11b+ Ly6C+ Ly6G+ myeloid-derived suppressor cells within regressing lesions. These results are consistent with a model whereby IL-12 triggers the maturation of tumor infiltrating myeloid-derived cells into competent APCs capable of cross-presenting tumor antigens. Licensed recognition of these antigens by effector T cells may in turn trigger the collapse of the tumor stroma and aid in the regression of large vascularized lesions.

NINDS
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Clinical Fellow
Clinical and Translational Research
Prevention of intraoperative cerebrospinal fluid leaks by lumbar drainage during transsphenoidal surgery for pituitary macroadenomas
Introduction: Cerebrospinal fluid (CSF) leak is a major complication of transsphenoidal surgery for pituitary tumors and can lead to headache, meningitis and neurologic sequelae. An intraoperative CSF leak is the only modifiable risk factor for postoperative leaks and occurs in up to 50% of cases. Although several techniques have been described for surgical repair when an intraoperative leak is noted, no technique has been proposed to prevent an intraoperative CSF leak. We postulated that intraoperative CSF drainage would diminish the tension on the arachnoid layer (enhancing its resistance to puncture, as in a partially deflated balloon), reduce the rate of intraoperative CSF leakage during surgery for larger tumors, and reduce both the need for surgical repair and the rate of postoperative leaks. Methods: Consecutive patients who underwent transsphenoidal surgery for pituitary macroadenomas (tumors greater than 1 cm in diameter) from 2007 to 2011 by a single surgeon were included. Results of 98 consecutive operations performed without intraoperative lumbar CSF drainage were compared with 27

consecutive cases in which a lumbar subarachnoid catheter was placed before surgery to drain 20 to 50 cc of CSF at the time of dural exposure. Results: CSF drainage reduced the rate of intraoperative CSF leak from 46% to 7% (odds ratio=0.09; P=0.0002). The rate of postoperative CSF leak was also reduced (5% to 1%), despite reducing the need for operative repair (33% to 3%, P=0.002). Superior extension of the tumor above the sella turcica, and towards the arachnoid, was not correlated with rate of intraoperative leak in the group with preoperative drainage. No patient experienced a lumbar drain-associated complication. Conclusions: CSF drainage during transsphenoidal surgery for macroadenomas reduces the rate of intraoperative CSF leak. This preventative measure obviated the need for autologous fat graft placement, postoperative lumbar drainage, and/or reoperation in most patients with macroadenomas. Given the minimal risk of complications with drainage, intraoperative lumbar CSF drainage should be considered before transsphenoidal surgery for macroadenomas.

NHGRI
Urick, Mary Ellen
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Clinical and Translational Research
PIK3R1 (p85-alpha), the Regulatory Subunit of PI3K, is Somatically Mutated at a High Frequency in Primary Endometrial Cancer
Purpose: Endometrial cancer kills more than 50,000 women worldwide each year. New therapeutic options are needed to treat women with advanced or recurrent cases of the disease. The phosphatidylinositol 3-kinase (PI3K)-AKT signal transduction pathway is often deregulated in cancer and is an important therapeutic target. The PI3K heterodimer consists of a p85 regulatory subunit, encoded by PIK3R1, and a p110 catalytic subunit, encoded by PIK3CA. The p85 subunit stabilizes and inhibits the p110 subunit. We recently reported a unique pattern of activating somatic mutations in p110 in endometrial tumors, with a high frequency of mutations in the p85 -interacting domains. We therefore hypothesized that disruption of p85 itself might be important in endometrial tumorigenesis. The purpose of this study was to determine the frequency of p85 mutations in endometrial tumors. Methods: We sequenced all coding exons from the DNA of 108 primary endometrial tumors. Somatic mutations were distinguished from germline polymorphisms by sequencing matched normal DNA. Wild-type p85, mutant p85, or vector controls were stably transfected in U2OS, an osteosarcoma cell line with low endogenous AKT phosphorylation. Binding to p110 and phosphorylation of AKT and S6 proteins were determined by immunoprecipitation and Western blotting. Results: We found a very high frequency of p85 mutation: 24% of endometrial tumors were mutated. Strikingly, over 90% of mutations, including a number of recurrent mutations, occurred in the p110 -interacting domains. The high frequency and nonrandom distribution of mutation suggested functional relevance. Therefore, we studied seven recurrent p85 mutants and found that five, including a positive control, retained the ability to bind p110 and increased phosphorylation of AKT and S6. Two truncating mutants, including a negative control, did not activate AKT or bind p110. Interestingly, these two mutations co-occur with p110 mutations. We therefore hypothesize that these two truncating mutants of p85 co-operate with p110 mutants and contribute to tumorigenesis via an AKT-independent mechanism. Conclusion: We have provided the first report of a high frequency of PIK3R1 (p85) mutation and a new mechanism of PI3K pathway activation in endometrial cancer. These findings are significant because they identify a new cohort of endometrial cancer patients that might respond to targeted therapies directed against the PI3K pathway.

NCI-CPFP
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Cultural Social and Behavioral Sciences

Accelerometer-based measures of active and sedentary behaviors in relation to breast cancer risk: The NCI Polish Breast Cancer Study.

BACKGROUND: Epidemiologic studies suggest that physical activity reduces breast cancer risk. However, studies have relied only on self-report and few have considered independent effects of sedentary time on risk. In the population-based NCI Polish Breast Cancer Study, we evaluated accelerometer measures of active and sedentary behavior in relation to breast cancer risk, with the aim of assessing whether these measures are better discriminators of risk than self-report. METHODS: This analysis includes 996 incident breast cancer cases and 1,164 controls, residents of Warsaw, Poland in 2000-2003. At the home visit (median=60 days postdiagnosis for cases), women ages 20-74 years were asked to wear an accelerometer (Actigraph 7164) for 7 days and complete a questionnaire including self-reported activity history. The accelerometers provided an objective measure of bodily movement (via an activity count) and captured patterns of time spent active and sedentary. Monitor values were averaged across valid wear days and summarized by quartiles (Q) of minutes spent per day in sedentary (0-99 counts), light (100-759 counts) and moderate-to-vigorous (760+ counts) behavior. Odds ratios (OR) and 95% confidence intervals (CI) were estimated using unconditional logistic regression. RESULTS: Time spent in moderate-to-vigorous activity was inversely associated with risk after adjustment for breast cancer risk factors, sedentary behavior and monitor wear time (OR Q4vsQ1: 0.38, 95%CI: 0.26-0.54; p-trend<.001). Increased sedentary time was related to increased risk, independent of moderate-to-vigorous activity (OR Q4vsQ1: 1.69, 95%CI: 1.16-2.45; p-trend<.01). These relationships were stronger than those previously observed with self-report. Restricting days since diagnosis and treatment yielded similar results. Associations did not differ by histology, tumor size, grade or nodal involvement. CONCLUSIONS: In this first breast cancer study of objective activity measures, increased moderate-to-vigorous activity appears to reduce breast cancer risk while time spent sedentary independently increases risk. Although the relations must be cautiously interpreted given the case-control design, there was little evidence that risk relations varied by clinical characteristics. Our findings suggest that physical activity may affect risk to a greater extent than previously hypothesized and are of importance given that activity is one of the few modifiable risk factors for breast cancer.

NIDA

Jobes, Michelle

Postdoctoral Fellow

Cultural Social and Behavioral Sciences

DRINKING AND DRUG USE FROM A PROSPECTIVE PERSPECTIVE

The relationship between alcohol and drug use has been described almost exclusively by retrospective means. Ecological Momentary Assessment (EMA) allows real-time collection of data on mood, activities, alcohol consumption and illicit drug use. EMA has provided insights into cigarette smoking, dietary habits and psychiatric disorders. Previously, we have used EMA to study precipitants of craving and use of cocaine and heroin. Here, I examine how craving and use of those drugs is related to alcohol consumption. The day-to-day experience of addiction and recovery was examined using EMA in a prospective, longitudinal cohort study of methadone-maintained heroin and cocaine users; individuals who met DSM criteria for alcohol abuse or dependence were excluded. Participants (n=114) carried handheld computers (PDAs: personal digital assistants) during waking hours for up to 25 weeks. Participants reported their locations, moods and activities in 2-5 randomly prompted (RP) entries per day, and they initiated an entry whenever they craved or used heroin or cocaine. Alcohol drinking was assessed in both types of entries. Repeated-measures logistic regression was used to assess the likelihood of drinking as a function of drug use vs. craving episodes vs. RP entries, and to assess the intensity of ongoing “background” craving in RP entries. Participants reported drinking alcohol in 1.6% of

RP entries. Frequency of drinking was 2.25 times higher in drug-craving episodes than in RP entries (p < .0001) and 7.7 times higher in drug-use episodes than in RP entries (p < .0001). Frequency of drinking was 3 times higher in drug-use episodes than in craving episodes (p < .0001). Within RP entries, the likelihood of drinking increased linearly with intensity of ongoing "background" drug craving (p < .0001). The association between alcohol and drug use previously established in retrospective studies was confirmed in this prospective EMA study. Even among participants with low baseline rates of alcohol consumption, alcohol was associated with drug craving and actual use. Drinking alcohol during drug-craving episodes and drug-use episodes was elevated over the base rates assessed by RPs. The likelihood of drinking alcohol increased significantly as the intensity of drug craving increased. The use of EMA enables us to conclude that these relationships are demonstrable in real time in users' normal environments.

NCI-CPFP
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Cultural Social and Behavioral Sciences
The influence of sedentary activity on the relationship between cigarette smoking and body weight: Results from the National Health and Nutrition Examination Survey (1999-2006)
Tobacco use and obesity are leading causes of death and disease in the U.S. In 2008, 20.6% of adults were current cigarette smokers and 33.8% were obese. Research has shown that current smokers have a lower body mass index (BMI) than never and former smokers. Thus, weight gain may be a barrier to smoking cessation. Smoking cessation benefits are well known, yet little research has examined factors that may minimize weight gain among former smokers in a national study. Sedentary activity (SA) is one such factor. Data from the National Health and Nutrition Examination Survey 1999-2006 were utilized to examine the relationship between smoking status, SA, and BMI. Smoking status was defined as being a current, former or never smoker. SA reflected average hours of daily TV and video use outside of work in the past 30 days (low: =1 hour; moderate: 2-hours; high: =4 hours). BMI was obtained through a physical exam. Individuals under 20 years of age or pregnant were excluded. A multistage probability design was utilized in order to obtain a nationally representative sample of the U.S. population. BMI was greater in former smokers (M=28.81, SE=.15) compared to never smokers (28.40, SE=.13) (t=-2.85, p=.0060); and greater in never smokers compared to current smokers was (27.18, SE=.12) (t=7.82, P<.0001). SA was lower in never smokers compared to former smokers (t=-8.88, p<.0001); and lower in former smokers compared to current smokers (t=-3.89, p=.0003). BMI and SA were significantly related such that those with less SA had a significantly lower BMI (F=44.43, p<.0001). A logistic regression tested the modifying effect of SA on the relationship between smoking status and obesity, controlling for the main effects of smoking, SA, gender, ethnicity, age, education, and survey year. The interaction of SA and smoking status significantly predicted obesity (F(4)=4.28, p=.0041). High SA current, former, and never smokers and moderate SA former and never smokers had significantly higher odds of being obese compared to low SA never smokers. Major changes in both smoking and obesity prevalence have occurred in the U.S. These results demonstrate that current smokers are more sedentary than former and never smokers. Post-cessation weight gain may be related to an unmasking of the negative effects of SA. Low SA former smokers may be less likely to experience cessation related weight gain. These findings have key implications for public health interventions.

NCI-DCCPS
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Cultural Social and Behavioral Sciences

Gaps in health information for cancer survivors: indicators of those most in need
Background: How to identify cancer survivors who need quality and timely health information is largely understudied. Interaction with the health care system and socio-demographic characteristics may predict unmet needs among cancer survivors. Methods: We examined health information needs in long-term survivors of breast, colorectal, endometrial, ovarian and prostate cancer from a population-based cross-sectional survey: FOllow-up Care Use among Survivors (FOCUS), which included 1582 survivors 4-14 years post-diagnosis. Endorsement of six health information needs categories (tests/treatments, side effects/symptoms, health promotion, interpersonal/emotional, insurance, and sexual functioning/fertility), total number of unmet needs, and confidence in obtaining health information were regressed on demographic variables, cancer site and recurrence, number of comorbid conditions, health insurance status, quality of recent follow-up care, receipt of treatment summaries, type of main follow-up doctor, and social support using multivariate models. Results: All information needs categories had moderate to high endorsement across cancer types (34-76%). In the multivariate models, survivors who were younger age, African American, and those with lower social support (P < 0.05) reported a significantly higher number of needs. African Americans, Hispanics, and Asian Americans expressed significantly more interpersonal/emotional and insurance information needs than non-Hispanic whites (P < 0.05). Survivors who did not receive a summary of their treatment but who would have liked one were more likely to indicate higher needs about tests/treatment (adjusted odds ratio: 1.88, 95% CI: 1.04-3.40) and side effects and symptoms (adjusted odds ratio: 2.74, 95% CI: 1.54-4.87) than those who had received a treatment summary. The number of health information needs endorsed was inversely related to the level of confidence (self-efficacy) in obtaining information (P < 0.05). Conclusion: Disparities in health information needs persist in long-term cancer survivors and are elevated particularly in those who are minorities, younger age, and those who did not receive a summary of their treatment. The association between a higher number of information needs and reduced self-efficacy suggests the need for better communication and post-treatment care practices that equip survivors with desired health information.

NIDDK
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Developmental Biology
Role of a mouse cortical-granule protease in the post-fertilization block to polyspermy
Survival of each mammalian species depends on fertilization, and control of reproduction remains a significant global human health issue. Much remains unknown about the molecular events that ensure that a single sperm fertilizes a single egg. Failure to do so results in polyspermy and embryonic death. Sperm bind to eggs but not to two-cell embryos, and a major defense against post-fertilization polyspermy is provided by the zona pellucida (ZP). The mouse ZP is composed of three glycoproteins, ZP1, ZP2 and ZP3. Following fertilization, ZP2 is cleaved by a protease thought to reside in cortical granules (CGs) located at the periphery of growing oocytes. Virtually nothing is known about the biology of CGs or their molecular contents in mammals. Upon egg activation initiated by gamete fusion, CGs migrate to the periphery and exocytose their contents which presumably diffuse through the ZP. Having biochemically characterized the proteolytic cleavage site of mouse ZP2, we have recently identified the first CG-specific protease (CGP) in mice. We have established a mouse line lacking CGP using DNA recombineering and embryonic stem cell technology to successfully target the single-copy gene encoding CGP. Mouse ZP2 is uncleaved following fertilization in CGP-null animals and sperm bind de novo to CGP-null two-cell embryos in which ZP2 remains intact. Thus, CGP regulates sperm-egg recognition by determining the cleavage status of ZP2. Additionally, the CGP has been fluorescently tagged, transiently expressed in CHO cells and analyzed on immunoblots with polyclonal antibody to

CGP as well as to GFP/mCherry. These CGP-fluorescent constructs have been microinjected into growing oocytes and demonstrate successful targeting to CGs by confocal microscopy. Using site-directed mutagenesis of these constructs, we will identify protein motifs essential for intracellular trafficking to CGs which will be confirmed with mouse transgenesis. We are also establishing a second transgenic mouse line expressing CGP fluorescently tagged with mCherry in order to rescue the CGP-null phenotype. Both the CGP null mutant and rescue mouse lines will be used for in vivo dissection of the intracellular trafficking of this protease and its role in the post-fertilization block to polyspermy. By characterizing these events in vivo and in vitro, these investigations will provide insight into the molecular mechanisms of fertilization with translational implications for reproductive medicine.

NCI-CCR
Huang, Bau-Lin
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Developmental Biology
A novel role for 5'HoxD genes in specifying early joint progenitors upstream of Gli3 and ß-Catenin
The evolutionarily conserved Hox gene clusters encode positional identity along the body axis of early vertebrate embryos. Yet their roles in specifying digit identities (thumb to pinky) are not clear. Deletion of 5' members of the HoxD cluster (HoxD11-13; 5'HoxD mutant) in mouse results in loss of the middle phalanges of each digit and fused joints, similar to human HoxD13 mutations phenotypes. We aim to elucidate the role of 5'HoxD genes in joint formation, a hallmark of digit identity. During digit formation, the expression of 5'HoxD and Gdf5, an early joint progenitor marker, surround the condensed digit precursors and presumptive joint regions (interzones). In 5'HoxD mutants, Gdf5 expression remained perichondrial and was only weakly and irregularly expressed in presumptive joint regions compared to sibling controls. Conditional deletion of a 5'HoxD allele specifically in joint progenitors using Gdf5Cre+/-;5'HoxDflox/flox mice did not produce abnormal joint phenotypes, indicating that 5'HoxD genes function earlier than Gdf5 in initiating joint formation. ß-Catenin expression in interzones of 5'Hoxd mutant digits was weak and diffuse, in contrast to the normal distinct expression, and Sox9, a master regulator of chondrogenesis, became expressed throughout the presumptive joint region, suggesting that 5'HoxD genes regulate joint cell fate determination. ß-Catenin-mediated canonical Wnt signaling is both necessary and sufficient for joint formation. Therefore, we used a tamoxifen-inducible Cre line to selectively express a stablized ß-Catenin allele in the 5'HoxD mutant limb bud, which rescued normal joint formation. To investigate a potential mechanism for this rescue, we assessed Gli3 levels. Gli3 is a major Hedgehog (Hh) signaling effector and a repressor of Hh targets in the absence of ligand. Gli3 can interact directly with 5'Hoxd proteins and also with ß-Catenin, antagonizing ß-Catenin activity. We found that Gli3 RNA was highly elevated in 5'Hoxd mutant limbs, and the level of Ptch1 (a direct Hh target) was reduced, suggesting that elevated Gli3 was predominantly the repressor form. In summary, we have identified a novel role of 5'HoxD proteins in the initiation of joint formation and propose that they free ß-Catenin from Gli3 antagonism via repressing Gli3 expression and may also sequester Gli3 by binding. We are further testing this model by examining both genetic and potential protein-protein interactions between 5'HoxD, Gli3, and ß-Catenin.

NCI-CCR
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Developmental Biology
Identification of a Novel Multipotent Cell Lineage in the Lung
Multiple cell phenotypes contribute to the structure and function of the lung. In the alveoli, type II cells secrete surfactant, whereas type I cells are responsible for gas exchange. In the airways, Clara cells are

progenitors for the epithelial lining, including ciliated cells, which eliminate excess mucus and harmful particles. In addition, the airway lining contains rare but important pulmonary neuroendocrine cells (PNECs) that regulate breathing and may contribute to carcinogenesis. In developing lungs, PNECs begin to differentiate prior to the other cells, but their histogenesis has remained elusive. Achaete-scute homolog 1 (Ascl1), a neural transcription factor critical for PNEC development, is detected early in embryonic lungs. We recently found that the Ascl1gene regulates stem cell marker expression in the lung. Hence, we asked whether Ascl1-defined cells (ADCs) function as progenitors in the lung. In this study, we used a novel in vivo lineage tracing approach to follow the fate of ADCs during lung development and injury repair. R26R-stop-lacZ (Rosa) reporter mice were crossed with Ascl1-CreERTM (AC) mice, in which the Ascl1 promoter drives the expression of inducible Cre recombinase. ADCs and their descendants are permanently labeled after tamoxifen (Tam) administration to Rosa dams at representative gestational dates. The labeled cells in fetal and adult lungs were characterized by immunohistochemistry using highly specific differentiation markers. Moreover, adult mice were given naphthalene (Nap), which kills Clara cells. Following Tam administration, only PNECs resistant to Nap were labeled as ADCs and the fate of labeled PNECs during epithelial repair was examined. In all experiments, littermates without Cre served as controls. Interestingly, ADCs labeled at embryonic day E9.5 gave rise to both airway (PNECs, Clara, and ciliated cells) and alveolar cells. However, ADCs labeled at E11.5 gave rise to airway cells only. In mature lungs following the Nap injury, a number of regenerating Clara cells were also labeled. We conclude, for the first time to our knowledge, that neuroendocrine (NE) cell lineage in the developing lung is not limited to maturing PNECs, but also includes progenitors for non-NE cells including Clara, ciliated, and type II cells. Further, NE lineage participates in injury repair of the airway lining.

NIDCD
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Visiting Fellow
Developmental Biology
Reciprocal rescue of sensory cilia defects by Bbs6 and Cep290 alleles
Ciliopathies are developmental disorders that arise due to defects in cilia biogenesis and function. In humans, ciliopathies are the cause of several congenital disorders that affect various sensory systems including the visual, auditory and olfactory systems. Thus, a better understanding of ciliopathies is critical for a better understanding of disease pathogenesis and provides an important insight into fundamental aspects of cell biology. Bardet-Biedel Syndrome (BBS) is an archetypical ciliopathy, caused by mutations in several genes involved in cilia biogenesis and function. One of the more common genes to be involved in BBS pathologies is BBS6 (MKKS). Leber congenital amaurosis (LCA) an autosomal recessive retinal dystrophy, is a form of ciliopathy that is caused by mutations in a gene CEP290 (also known as NPHP6 or BBS14). The CEP290 gene has been implicated in a large percentage of ciliopathies and as such is expected to be one of the key contributors to disease pathogenesis. Little is known about the function of either BBS6 or CEP290, nor how these proteins interact with other cilia-related proteins complexes. Our initial finding of variants of BBS6 in LCA patients prompted us to study their interactions in mouse models and led to an exploration of epistatic interactions between CEP290 and BBS6. We found that the DSD domain (Deleted in Sensory Dystrophy) of CEP290, which is deleted in a mouse model (Cep290rd16) of LCA, directly interacts with BBS6, and that pathogenic variants of BBS6 disrupt this interaction. Mice with either Cep290rd16/rd16 or Bbs6ko/ko genotypes exhibit structural and functional auditory, photoreceptor, and olfactory deficits. Surprisingly, in contrast to the defects seen in either single mutant mouse, mice double mutant for both these genes actually show a degree of functional and morphological rescue in all three sensory systems. Morphological analysis suggests that improved ciliogenesis forms the mechanistic basis for this functional rescue. Thus, our data demonstrate

reciprocal modifier effects between CEP290 and BBS6 that provides insight into the complex etiology of human diseases and in the regulation of cilia formation and function.

NHGRI
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Developmental Biology
Loss of Gas in mesenchymal progenitor cells leads to heterotopic ossification resulting from dysregulated hedgehog signaling
Heterotopic ossification (HO), the inappropriate presence of bone outside of the skeleton, is a major complication of many traumas (e.g. war time extremity trauma, brain injury, hip/knee surgery), and is also seen in certain genetic diseases. One such disease is Progressive Osseous Heteroplasia (POH), which is characterized by subcutaneous HO progressing into deep connective tissues. POH is associated with loss of function mutations in GNAS, which encodes the stimulatory G protein subunit Gas. Like other forms of HO, the pathogenesis of POH is unknown and suitable in vitro and in vivo models have yet to be developed. Using mouse genetics we have removed Gas from mesenchymal progenitors cells and generated an in vivo model of POH. Like human patients, mutant mice develop subcutaneous HO that progresses into deep connective tissues leading to joint fusions and limb immobility. Using this mouse model we set out to identify the pathophysiology of POH. It is known that hedgehog signaling promotes mesenchymal progenitor cell differentiation towards the osteoblast lineage, the cell-type that produces bone. As Gas is a major activator of protein kinase A (PKA) and PKA is a potent inhibitor of the hedgehog signaling pathway, we hypothesized that the hedgehog pathway was inappropriately upregulated in POH. This was confirmed in our mouse model as western blotting demonstrated enhanced processing of the Gli3 transcription factor to the activator form in affected tissues. Similarly, qRT-PCR and in situ hybridization showed dramatic elevation of hedgehog target genes. Further evidence supporting this hypothesis included a strong genetic interaction in which loss of one copy of the hedgehog receptor Patched1 synergized with the Gas mutant mice to cause embryonic lethality with polydactyly and exencephaly. Also, activation of the hedgehog pathway in mesenchymal cells in vivo was sufficient to cause HO in mice. Finally, we demonstrated that an antagonist of the hedgehog pathway, arsenic trioxide, could inhibit HO formation in our mouse model. Our work demonstrates for the first time that inappropriate up-regulation of the hedgehog signaling pathway causes some forms of HO and that pathway antagonists may be of use in its treatment.

NIEHS
Hesse, Jill
Postdoctoral Fellow
DNA-binding Proteins/Receptors and DNA Repair
Identification and Discovery of DNA Damage-Induced microRNA Expression Changes by Microarray and Next-Generation Sequencing in Wild-Type and ATM-Deficient Human Mammary Epithelial Cells
MicroRNAs (miRNA) have been identified as important regulators of post-transcriptional gene expression and are critical in development, cell signaling pathways, and cancer progression. While many functions of miRNAs have been identified, it is far from clear the totality of roles these miRNAs are playing in cellular functions. The Ataxia-Telangictasia Mutated (ATM) gene product is a critical PI3-kinase related protein that has been shown to regulate DNA damage and cell cycle checkpoint responses. Individuals with deficiencies in ATM have severe ataxia and immunological problems and are predisposed to developing lympho-proliferative and breast cancers. Here we investigate the miRNA expression profile in response to both DNA damage induced by ionizing radiation (IR) and loss of ATM functionality. We utilize isogenic ATM wild type and ATM-deficient human mammary epithelial cell

cultures (HME-CCs) in order to investigate the normal and ATM-dependent mammary epithelial response to DNA damage by investigating mRNA expression responses as well as to investigate miRNA expression, with the ultimate goal of identifying profile signatures to identify IR-sensitive cells. We utilized two different methods to evaluate miRNA expression in HME-CCs, microarray and Next Generation sequencing. We are investigating both the best mechanism for evaluation of miRNAs as well as investigating the targets of statistically significant miRNAs by comparing the changes in expression of the miRNAs with changes in global mRNA gene expression in the same cells, in order to increase our understanding of the role of miRNA in DNA damage responses. We believe that direct comparison of the microarray and Next Generation sequence data will allow the most complete picture of miRNA regulation in response to DNA damage. Preliminary data analysis reveals both ATM-dependent and ATM-independent miRNA expression changes in response to DNA damage as well as ATM-dependent regulation of miRNA expression in the untreated conditions. Additionally, by exploiting the Next Generation sequencing technologies, we have identified possible novel DNA damage responsive small and miRNAs.

NIDDK
Hwang, Ling Chin
Visiting Fellow
DNA-binding Proteins/Receptors and DNA Repair
Dynamic self-organization of bacterial DNA segregation machinery in cell-free reaction
DNA segregation is a vital process that ensures the faithful transmission of genetic material during cell division. Eukaryotes use the polymerization/depolymerization of tubulin to separate chromosomes. In bacteria, plasmids have evolved segregation machineries to partition replicated DNA to the daughter cells. P1 phage lysogenizes as a low-copy-number plasmid in Escherichia coli. It encodes only three components for this reaction: a parS sequence acting as the partition site and two proteins, ParA and ParB. Previous studies showed ParB binds to parS, forming a partition complex. ParA, an ATPase and an ATP-dependent DNA-binding protein is thought to couple the ATP hydrolysis energy to drive plasmid motion. The non-specific DNA binding activity of ParA has been proposed to exploit the bacterial nucleoid as the reference scaffold for plasmid motion. However, how these system components work together to drive plasmid segregation remains unclear. To elucidate the mechanism of P1 plasmid partition, we reconstituted the partition system in vitro and visualized protein dynamics with total-internal-reflection-fluorescence microscopy. We coated a flow cell surface with non-specific DNA to mimic the nucleoid surface. ParA-GFP fusion was incubated with ATP, ParB, and fluorescence-labeled parS plasmid and flowed into the flow cell. ParA and ParB bound to the DNA-coated surface of the flow cell. The partition complexes containing preassembled ParA, ParB and parS plasmid also anchored themselves to the protein-coated DNA surface. ParA disassembled from the partition complex in response to the ATPase stimulation by ParB. As the ParA content of the partition complex decreased, we observed vigorous Brownian motion of the plasmid before detaching from the DNA-coated surface, leaving the surface area devoid of ParA. ParA quickly refilled these voids afterwards. In the presence of non-hydrolysable ATP analogue, the complexes were immobile on the DNA-coated surface. ParA mutants, defective in ATP-binding or hydrolysis failed to form the partition complex or formed immobile complexes. FRAP experiments showed highly dynamic protein-exchange on the DNA-coated surface and in the partition complex. Our in vitro reconstituted plasmid partition system is a powerful tool to investigate the reaction mechanism. We propose a diffusion-ratchet model for plasmid segregation based on our results. This model represents a paradigm shift in our view of the cell division process in bacteria.

NCI-CCR

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Visiting Fellow
DNA-binding Proteins/Receptors and DNA Repair
Understanding the role of CtIP in DSB repair
The repair of double strand breaks (DSBs) is essential for cell survival, and unprocessed breaks can promote genomic instability and cancer. The main pathways that repair DNA breaks include non homologous end-joining (NHEJ) and the homologous recombination (HR). A critical initiating event for HR is resection, in which DSB ends are processed to generate 3' single-strand DNA (ssDNA) overhangs which subsequently invade the homologous template strand. The factors that are critical for resection include nucleases which process the ends, and other proteins such as BRCA1, CtIP and ATM whose functions remain unclear. Although CtIP does not contain any known nuclease domains, it has been suggested to be required for the DSB end resection. In this study we investigated the physiological role of CtIP by analyzing genomic stability in CtIP deficient systems. In mice, the homozygous disruption of CtIP is embryonically lethal, therefore we conditionally knocked out the gene in B cells. Surprisingly, CtIP-deficient cells were able to proliferate, but they showed increased level of genomic instability, measured as a higher number of breaks and complex rearrangements of chromosomes compared to WT cells. The spontaneous accumulation of chromosome aberrations triggered persistent damage signaling, ultimately leading to increased cell death of CtIP-deficient cells. The loss of CtIP also resulted in decreased generation of ssDNA ends supporting the role of CtIP in promoting DNA resection and HR. We have shown that the non homologous end joining protein 53BP1 blocks resection by preventing access of nucleases to chromatin. The deletion of 53BP1 rescues homologous recombination in BRCA1-deficient cells. Therefore we asked whether the absence 53BP1 could also rescue the genomic instability of CtIP deficient cells by allowing the access of nucleases to the broken chromatin to promote resection and therefore efficient HR. CtIP/53BP1 double deficient cells showed elevated level of genomic instability which suggested that the HR pathway was not restored. Indeed, the loss of 53BP1 did not rescue the defect in resection observed in CtIP depleted cells. Our work, therefore, supports the role of CtIP but not Brca1 as controlling the nucleases that drives the DSB end resection necessary to promote efficient HR and maintain genome integrity.

NIA
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Postdoctoral Fellow
DNA-binding Proteins/Receptors and DNA Repair
Recruitment and retention of RecQ5 at laser induced damage sites and its possible association with WRN syndrome helicase.
RecQ helicases are conserved through out the evolution and play a very important role in the maintenance of genomic stability. There are five RecQ helicases in humans, three of which are associated with cancer and premature aging syndromes, BLM, WRN and RecQ4. RecQ5 plays an important role in homologous recombination (HR), in the removal of Rad51 filaments, and has been shown to associate with RNA pol II, suggesting an involvement in transcription. Following the role of RecQ5 in HR, we wanted to study the recruitment of RecQ5 to double strand breaks (DSBs), and further investigate if there is any functional interplay between RecQ helicases. Stable RecQ5 KD cells were generated by lentivirus constructs. RecQ5 KD cells are sensitive to IR, and persistent 53 BP1 foci were observed compared to scrambled cells implicating inefficient DSB repair. This present study focused on the recruitment and retention of GFP RecQ5 to laser induced DNA damage sites at 435 nm, as well as its endogenous association. First, we showed that the helicase activity of RecQ5 is not essential as an ATPase dead mutant gets recruited to laser induced DSBs as well as WT RecQ5. The recruitment kinetics of RecQ5 is studied and similar to that of other RecQ helicases. To determine if RecQ5's recruitment and

retention at DNA damage sites is influenced by any of the other RecQ helicases; we tested RecQ5 recruitment in WRN, BLM or RecQ4 deficient cell backgrounds. The recruitment of RecQ5 was independent of the presence of other RecQ helicases. Next, we evaluated how long RecQ5 stayed at the laser induced damage. Interestingly, GFP RecQ5 stayed longer at the damage sites in the absence of BLM & WRN but not RecQ4, thus indicating that these proteins may have some overlapping functions. RecQ5 might associate with unrepaired lesions that are persistent in the absence of BLM & WRN. Further, we observe that endogenous RecQ5 is over expressed in cells lacking BLM but not WRN, which also indicates that RecQ5 may be needed to compensate for the loss of BLM. Interestingly, RecQ5 specifically stimulates the helicase activity of WRN but not BLM on a fork duplex, suggesting a possible association of RecQ5 and WRN, presumably in the repair of stalled replication forks. This functional interplay between the RecQ helicases, is very important to study, as to how these RecQ helicases co-operate with each other, by their over lapping functions or their association in the maintenance of genomic stability.

NIA
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Postdoctoral Fellow
DNA-binding Proteins/Receptors and DNA Repair
RecQL5 cooperates with Topoisomerase II alpha in DNA decatenation and cell cycle progression.
DNA decatenation mediated by Topoisomerase II is required to separate the interlinked sister chromatids post replication. SGS1, a yeast homologue of the human RecQ family of helicases has been shown to interact with Topoisomerase II and play a role in chromosome segregation. RecQL5 is one of the five human members of the RecQ helicase family. The role of human RecQL5 is significantly understudied, though it has been shown that deficiency in RecQL5 homologue significantly reduces the lifespan of Caenorhabditis elegans and RecQL5 knockout mice show higher cancer incidence. To further investigate the role of RecQL5, we generated stable knockdowns of RecQL5 in cultured human cell systems, in both primary and transformed cells. Relative to Scramble control cells, cells harboring stable depletions of RecQL5 displayed slow proliferation rate, a G2/M cell cycle arrest and late S phase cycling defects. Metaphase spreads generated from RecQL5 depleted cells exhibited high under-condensed and entangled chromosomes, similar to a previously observed phenotype generated when Topoisomerase II catalytic activity is inhibited. We therefore hypothesised that RecQL5 depletion generated a Topoisomerase II dysfunction. Reciprocal immunoprecipitations from cell extracts and pull down of pure proteins suggested a direct interaction of RecQL5 with Topoisomerase IIa. Functionally, RecQL5 stimulated the decatenation activity of Topoisomerase IIa and RecQL5 depleted cell extracts showed decreased decatenation activity. Consistent with our results, RecQL5 colocalized with Topoisomerase IIa during late S and G2 phases of cell cycle. Further, RecQL5 depleted cells activated a G2 checkpoint and undergo apoptosis. Based on these observations, we propose a model in which the interaction between RecQL5 and Topoisomerase IIa plays a putative role in resolving torsional and replicative stress associated with termination of replication. In this model, RecQL5 helicase could resolve the steric constraints that arise due to the formation of positive supercoils during convergence of the opposite replication forks on a linear chromosome. By unwinding the duplex DNA and converting positive supercoils into interlocked catenates, RecQL5 would provide access for Topoisomerase IIa, which could then resolve the daughter strands. In conclusion, these results reveal an important role for RecQL5 in the maintenance of genomic stability and a novel mechanistic insight into the decatenation process.

NIA
Sykora, Peter
Visiting Fellow

DNA-binding Proteins/Receptors and DNA Repair

Aprataxin localizes to mitochondria and preserves mitochondrial function

The neurodegenerative disorder ataxia with oculomotor apraxia 1 (AOA1), causes progressive reduction of cerebellar volume resulting in sustained loss of motor control. AOA1 is caused by mutation in the APTX gene, encoding the DNA stand break repair protein aprataxin. A confounding aspect of AOA1 research is that unlike patient's afflicted with mutation in other DNA repair proteins; AOA1 patients are not sensitive to DNA damaging agents nor show an increase in cancer incidence. The similarity of AOA1 to neurodegerative disorders caused by mitochondrial DNA (mtDNA) repair defects lead us to question whether AOA1 may be the result of mitochondrial dysfunction. We report that aprataxin localizes to the mitochondria, confirmed by western analysis of mitochondrial cell fractions and by comparing the immunofluorescence co-localization of a mitochondrial tracking dye and aprataxin antibodies. Further, using a GFP tag, we identified an N-terminal amino acid sequence that targeted certain isoforms of the protein to this intracellular compartment. Transcripts encoding this unique N-terminal stretch were identified in all major human brain regions, with highest production in the cerebellum; the primary region of AOA1 related atrophy. To recapitulate the reduced levels of aprataxin in AOA1 patients and investigate mitochondrial impairment, we used a lenti viral delivered shRNA construct resulting in the stable depletion of aprataxin in mitotic and post-mitotic human SH-SY5Y neuroblastoma cells and primary skeletal muscle myoblasts. The reduction in aprataxin caused severe mitochondrial dysfunction, revealed by a decrease in citrate synthase activity, a measure of intact mitochondria, and also mtDNA copy number, both compared to scrambled shRNA controls. Using fluorescent based probes we report an increase in reactive oxygen species and the breakdown of mitochondrial networking in the aprataxin depleted cells. Analysis of DNA integrity using long Q-PCR, showed that mtDNA, not nuclear DNA, had high levels of background DNA damage upon aprataxin knockdown. Also, using a mutagenesis assay we confirm that aprataxin deficiency results in an increased rate of mtDNA mutagenesis, suggesting a direct role for the enzyme in mtDNA processing. Our research is the first to describe a mitochondrial function for aprataxin and highlights the critical role aprataxin plays in maintaining mitochondrial integrity. These findings provide new avenues for the treatment of patients with AOA1.

NIA

Chen, Zhike

Visiting Fellow

Endocrinology

Taste Precursor Cells, Potential Substitutes for Pancreatic ß Cells in Type 1 Diabetes

Type 1 diabetes results from autoimmune destruction of pancreatic ß cells, usually leading to absolute insulin deficiency. Pancreas and islet transplantations represent cell-based strategies for the treatment of type 1 diabetes. However, they are limited both by the scarcity of donor pancreata and immunologic rejection. Therefore, it would be helpful to the field to have an alternate cell-based therapy. Here, we illustrate a candidate that may serve as a novel source for ß cells. Pdx1, Ngn3 and MafA are three crucial transcription factors for insulin production. Our immunohistochemistry and PCR data showed that these three factors also exist in taste cells of mouse tongue. Moreover, the taste precursor cells have the same endodermal origin as ß cells. Hence, we hypothesized that taste precursor cells, which in vivo have the capability to differentiate into all four taste cell types, have the potential to be differentiated into ß cells. We isolated mouse taste precursor cells by enzyme digestion, and cultured the primary cells in vitro. Immunofluorescence imaging showed that sonic hedgehog homologue (Shh) and prospero-related homeobox (Prox1), markers of taste precursor cells, were expressed in the primary cells, proving that we successfully isolated and cultured the taste precursor cells. These cells were further identified by flow cytometry using the Aldefluor fluorescent reagent. Next, we developed a protocol to differentiate taste precursor cells into ß cells in vitro by manipulating TGFß, Hedgehog, PI3K, and Notch signaling pathways.

The primary cells were cultured with a group of selected factors, including Activin A, basic FGF, Exendin-4, for four weeks. We found that the gene expression of key ß cell transcription factors (Pdx1, Ngn3, Mafa, NeuroD) increased by 20-fold, and insulin 1 and insulin 2 genes by 50- and 35-fold, respectively, as compared with control cells, which were cultured in the same medium without the above factors. Furthermore, immunofluorescent staining confirmed the presence of C-peptide in these cells, indicating the synthesis of endogenous insulin. In conclusion, we successfully differentiated the taste precursor cells into insulin-containing cells. The results of our current work sheds a light on the possibility of using taste precursor cells to replace ß cell function and may contribute to a cure for type 1 diabetes.

NICHD
Fujimoto, Kenta
Visiting Fellow
Endocrinology
Transcription of thyroid hormone co-activator PRMT1 is regulated by a thyroid hormone-induced transcription factor c-MYC during Xenopus intestinal remodeling
Thyroid hormone (T3) plays a major role in tissue remodeling and organogenesis during postembryonic development throughout vertebrates. T3 deficiency causes a number of developmental abnormalities, such as cretinism in human. Unfortunately, understanding the underlying mechanims has been hampered by the difficulty to study gene regulation by T3 in uterus-enclosed mammalian embryos. Here we use the metamorphosis of amphibian Xenopus laevis as a model to study T3 function during development. During metamorphosis, the digestive tract is remodeled from the larval to adult form and the adult epithelial stem/progenitor cells are developed de novo from the larval epithelium through yet known mechanism. It has been shown that T3 exerts its metamorphic effects through T3 receptor (TR)-mediated transcriptional modulation of downstream genes. We have shown that the TR-coactivator protein arginine methyltransferase 1 (PRMT1) is upregulated in a small number of larval epithelial cells that appear to give rise to the adult stem cells and that and PRMT1 upregulation is the earliest molecular marker known to date. Surprisingly, stem cell specific expression of PRMT1 is conserved during zebrafish and mouse intestinal development, suggesting that PRMT1 function is conserved. To analyze how PRMT1 is specifically upregulated in the stem cells, we first characterized the X. laevis PRMT1 promoter in CaCo-2 cells, a human intestinal epithelial cell line with intestinal stem cell characters. We have identified evolutionally conserved elements in the promoter and in the first intron involved in the PRMT1 transcriptional regulation using luciferase reporter assays. Our findings suggest the involvement of the c-MYC transcription factor, which is known to be important for stem cells and cell proliferation. We hypothesized T3 induces c-MYC to regulate PRMT1 transcription. Interestingly, qRT-PCR analysis showed that c-MYC is upregulated during natural and T3-induced metamorphosis prior to PRMT1 upregulation in the intestine, and chromatin immunoprecipitation assay indicated TR binds to a putative T3 response element in c-MYC gene of X. tropicalis, its genome has been sequenced recently. Furthermore, we found c-MYC regulates the promoter through the conserved binding site in PMRT1 gene through promoter mutational studies. Thus, our findings suggest that T3 induces c-MYC directly in the intestine, which in turn enhances PRMT1 expression through the cis-element in the first intron.

NCI-CCR
Jiang, Changtao
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Endocrinology
Inhibition of hypoxia-inducible factor 1 in adipocytes protects against high-fat-diet-induced obesity and insulin resistance

Obesity, insulin resistance and type 2 diabetes mellitus are a tightly correlated cluster of metabolic disorders in which adipose is one of the first affected tissues. In the obese mouse model, hypoxia occurs specifically in adipose tissue and the response to adipose hypoxia may lead to insulin resistance. Hypoxia-inducible factor 1 alpha (HIF1alpha) is the main signal mediator of the hypoxia response in adipose tissue. To investigate the role of hypoxia in the development of obesity and insulin resistance, mice with adipocyte-specific targeted disruption of the HIF1alpha were generated using the Cre/LoxP system. After 12 weeks of high-fat diet (HFD) feeding, adipocyte-specific HIF1alpha knockout mice had reduced fat formation and were protected from HFD-induced obesity and insulin resistance as compared with similarly fed wild-type mice. Indirect calorimetry revealed that resting and total oxygen consumption adjusted to body weight were significantly increased both at 24 Centigrade and 30 Centigrade, and resting and total RER at 30 Centigrade were reduced in adipocyte-specific HIF1alpha knockout mice. These changes could contribute to the decrease in weight gain on a HFD as compared to wild-type controls. Glucose tolerance and insulin tolerance tests revealed that glucose tolerance and insulin sensitivity began to be improved in adipocyte-specific HIF1alpha knockout mice from 4 weeks on a HFD. HIF1alpha deficiency in adipocytes improved insulin signaling pathways in adipose tissue, liver and skeletal muscle. The improved insulin resistance was associated with increased expression and secretion of the adiponectin, an adipokine secreted by adipocytes that modulates a number of metabolic processes, including hepatic glucose metabolism and fatty acid catabolism. In vivo and in vitro studies revealed that the suppressor of cytokine signaling 3 (Socs3) is a direct target gene of HIF1alpha. HIF1alpha suppresses the expression of adiponectin through the SOCS3-signal transducer and activator of transcription 3 (STAT3) pathway that regulates the adiponectin gene. Finally, acriflavine, a specific inhibitor of HIF1alpha, caused decreased body weight and increased insulin sensitivity as compared to vehicle-treated control mice on a HFD. These studies revealed that inhibition of HIF1 in adipose tissue ameliorates type 2 diabetes and provide compelling evidence that HIF1 could be a potential therapeutic target for obesity and type 2 diabetes.

NIEHS
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Postdoctoral Fellow
Endocrinology
The Role of Glis3 in the Development of Functional Pancreatic Beta-cells and Diabetes
Gli-similar (GLIS) 3 belongs to a subfamily of Krüppel-like zinc transcription factors that are related to members of the Gli and Zic family. In recent years, several genes including GLIS3 have been associated with risk for type-1 and type-2 diabetes and genetic aberrations in the GLIS3 gene have been associated with a syndrome characterized by neonatal diabetes and congenital hypothyroidism. We have shown that Glis3 KO mice die at post-natal day 3 from neonatal diabetes as evidenced by hyperglycemia and hypoinsulinemia. This phenotype is due to a dramatic loss of insulin-secreting beta-cells in contrast to a smaller relative loss of other endocrine cell types. In addition, Glis3 regulates insulin gene expression in mature beta-cells, indicating that Glis3 plays a key role in both the development and function of mature pancreatic beta-cells. However, the spatial and temporal expression of Glis3 during pancreas development and the mechanism by which Glis3 contributes to the development and maintenance of functional beta-cells are unknown. We have generated two pancreas-specific Glis3 KO mouse models. Glis3 fx/fx mice were crossed with mice expressing cre-recombinase under the control of the Pdx or Insulin gene promoters, expressed at early and late time-points during pancreas development, in order to generate pancreas- and beta-cell-specific KO models respectively. Pdx(Glis3) KO mice develop a delayed-onset diabetic phenotype at 2-3 months of age as evidenced by hyperglycemia, hypoinsulinemia, and loss of white adipose tissue whereas Ins(Glis3) KO mice do not develop overt diabetes. Gene expression profiling and immunofluorescence analysis demonstrated that the expression

of insulin was significantly decreased at 2 months, but not at 2 weeks, of age in Pdx(Glis3) KO mice. The expression of other pancreatic hormones and transcription factors important for endocrine cell development, including Ngn3 and Pdx1, was not significantly different than that of WT littermates, indicating that, unlike in the whole-body KO, there is not a significant loss of beta-cells in the Pdx(Glis3) KO mice. The Pdx(Glis3) KO mice appear to provide an excellent model to examine various therapeutic strategies for diabetes. Our study shows that Glis3 has multiple critical functions in the pancreas and suggests that Glis3 may provide a new therapeutic target to intervene in diabetes.

NIEHS
Winuthayanon, Wipawee
Research Fellow
Endocrinology
Role of epithelial estrogen receptor alpha in the oviduct during fertilization and embryo development
Mammalian fertilization and preimplantation embryo development occur in the oviduct within a microenvironment strictly controlled by steroid hormones. Changes in oviductal cytology in each cell compartment are the most apparent during the estrous stage of the ovarian cycle, immediately prior to fertilization. The goal of this study was to elucidate the role of estrogen receptor alpha (ERa) in oviduct epithelium during fertilization and preimplantation embryo development. We previously generated a conditional knockout (cKO) mouse lacking ERa in female reproductive tract epithelium by crossing Wnt7a-Cre with Esr1-floxed mice. The cKO females were completely infertile in part because of inability to achieve uterine receptivity. To determine if oviductal dysfunction also contributed to their infertility, we first documented by immunohistochemistry that ERa was not expressed in oviductal epithelium of the cKO mice. We then examined ovulated oocytes and evaluated preimplantation embryo development in vivo by flushing embryos from the oviduct and uterus on days 1, 2 and 4 of pregnancy. The cKO mice ovulated similar numbers of morphologically normal appearing eggs as control littermates, and one-cell stage embryos were flushed from the oviduct on pregnancy day 1. However, there were no 2-cell embryos or blastocysts on day 2 or day 4 of pregnancy, respectively, although a few fragmented embryos and empty zona pellucidae were observed. When the one-cell embryos were cultured in vitro, very few embryos collected from cKO oviducts progressed to the expanded blastocyst stage whereas most embryos collected from control oviducts developed into hatching blastocysts. These results indicate that lack of ERa in the oviductal epithelium results in alterations in the oviductal microenvironment that completely disrupt preimplantation embryo development beginning at the one-cell stage. This is a highly novel and unexpected regulatory phenomenon of epithelial ERa in the female reproductive tract that will provide information important for understanding mechanistic actions of estrogenic environmental chemicals and their impact on very early mammalian pregnancy as well as the roles of ERa in human fertility.

NCI-DCEG
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Epidemiology/Biostatistics - Etiology, Risk, and Prevention
Genetic Variation in DNA Repair Genes, Pesticide Exposure, and Prostate Cancer Risk
BACKGROUND: DNA damage plays a critical role in carcinogenesis. Various pesticide exposures have been associated with increased DNA damage, suggesting that this mechanism might contribute to pesticide-associated cancer risk. OBJECTIVE: For the first time, we evaluated interactions between pesticide exposures and genetic variation in two DNA repair pathways thought to be important in repairing pesticide-induced DNA damage, base excision repair (BER) and nucleotide excision repair (NER), with respect to prostate cancer. METHODS: We examined 39 pesticides with 394 tag single

nucleotide polymorphisms (SNPs) for 31 BER genes and 324 tag SNPs for 27 NER genes among 776 prostate cancer cases and 1,444 male controls in a nested case-control study of white Agricultural Health Study pesticide applicators. We characterized cumulative exposure to each pesticide into three categories (none/low/high) based on lifetime days of use weighted to an intensity score using information reported at enrollment. We assumed the dominant genetic model for SNPs. We used likelihood ratio tests from logistic regression models to estimate interaction P-values, and the False Discovery Rate (FDR) method to adjust for multiple comparisons. RESULTS: We identified several BER and NER SNPs that modified pesticide associations with prostate cancer, such that men in one genotype group exhibited a monotonic increase in risk with increasing pesticide exposure and men in the other group exhibited no significant change in risk. Several of these interactions also met FDR less than 0.2. Fonofos was associated with increased prostate cancer risk among men carrying the variant T allele for NEIL3 rs1983132 (Odds Ratio (OR) for high versus no fonofos use=3.25; 95% Confidence Interval (CI): 1.78-5.92; Pinteract=9.3x10-6; FDR-adjusted P=0.01) or the variant A allele for ERCC1 rs2298881 (OR=2.98; 95% CI: 1.65-5.39; Pinteract=3.6x10-4; FDR-adjusted P=0.11). Carbofuran was associated with increased risk among men carrying the homozygous wild-type TT genotype for two highly correlated SNPs (r2=1.0) tagging CDK7, rs11744596 and rs2932778 (for rs11744596, OR for high versus no carbofuran use=2.01; 95% CI: 1.31-3.10; Pinteract=7.2x10-4; FDR-adjusted P=0.09). In contrast, associations were null among men with other genotypes at these loci. CONCLUSION: Our findings suggest that genetic variation in the BER and NER pathways might influence susceptibility to prostate cancer among those exposed to certain pesticides.

NCI-DCEG
Hofmann, Jonathan
Postdoctoral Fellow
Epidemiology/Biostatistics - Etiology, Risk, and Prevention
Pre-existing kidney disorders and risk of renal cell carcinoma: results from a population-based case-control study of Caucasians and African Americans
Background: In the United States, the incidence of renal cell carcinoma (RCC) is higher among African Americans (AA) than among Caucasians. However, few studies have investigated potential risk factors that may contribute to racial disparities in RCC incidence. Established risk factors for RCC include smoking, obesity, and hypertension. Beyond these factors the etiology of RCC remains largely unknown, though previous observations suggest that patients with end-stage renal disease on long-term dialysis may be at increased risk of developing RCC. Methods: We conducted an investigation of RCC risk in relation to pre-existing kidney disorders in a population-based case-control study of AAs and Caucasians in the metropolitan regions of Chicago and Detroit. Personal interviews were conducted to obtain information on history of kidney disorders and other covariates from 1,217 RCC cases (361 AAs and 856 Caucasians) and 1,235 controls (523 AAs and 712 Caucasians). Odds ratios (OR) and 95% confidence intervals (CI) were estimated using unconditional logistic regression models adjusted for age, sex, race, level of education, smoking status, body mass index, history of hypertension, family history of cancer, and study center. Analyses stratified by race and time since kidney disease diagnosis were also performed. Results: A statistically significant increased risk of RCC was observed among subjects who had been previously diagnosed with chronic renal failure (OR 4.7, 95% CI 2.2-10.1, P<0.001) or were on dialysis (OR 18.0, 95% CI 3.6-91.1, P=0.001). Stronger associations were observed among subjects who had renal failure or were on dialysis 10 or more years prior to RCC diagnosis. The association between chronic renal failure and RCC was considerably stronger among AAs than among Caucasians (ORs of 8.7 and 2.0, respectively; P-interaction=0.03). Among AAs, history of kidney stones, kidney cysts, and nephrotic syndrome were all associated with an elevated risk of RCC (OR>=2.0), though these findings were not statistically significant. Conclusions: The results of this large population-based study

suggest that chronic renal failure and dialysis may be important risk factors for RCC development. Our findings of differences in risk estimates by race, to our knowledge the first such report, may explain in part the higher incidence of RCC among AAs. Replication of these findings is needed.

NCI-CPFP
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Epidemiology/Biostatistics - Etiology, Risk, and Prevention
The association of diabetes with cancer incidence and mortality in the NIH-AARP study
Objective: Incidence rates for diabetes and cancer are increasing globally. Epidemiologic studies have observed increased risk between diabetes and several cancers, including colorectal, liver, and pancreatic. Yet, the association of diabetes with other cancers and with cancer overall remains unclear. Thus, we evaluated the association of diabetes with cancer incidence and mortality in the prospective NIH-AARP Diet and Health Study. Methods: Our analysis included 295,287 men and 199,665 women who resided in one of eight states (CA, FL, GA, LA, MI, NC, NJ, and PA) at baseline (1995-1996). Diabetes was assessed by self-report. Hazard ratios (HR) and 95% confidence intervals (95% CI) were estimated from Cox proportional hazards models adjusted for age, education, BMI, smoking status, self-reported health status, physical activity, vitamin supplement use, intake of alcohol, fruits, vegetables and meat, family history of cancer, and among women, menopausal hormonal therapy. Results: After 11 years of follow-up, 55,888 men and 26,364 women were diagnosed with incident cancer; 13,037 men and 6,474 women died of cancer. We observed a modest inverse association between diabetes and total incident cancer in men (HR=0.96, 95% CI: 0.93-0.99) and a modest positive association in women (1.08, 1.03-1.13). Inverse associations in men stemmed from prostate cancer (n=23,483 cases, 42% of incident cancers; 0.74, 0.70-0.78). After excluding prostate cancer, diabetes became positively associated with cancer in men (1.08, 1.05-1.13). For total cancer mortality, we observed a positive association in men (1.17, 1.11-1.24) and women (1.11, 1.01-1.22). When evaluated by site, diabetes was positively associated with colon (1.16, 1.05-1.29; 1.31, 1.12-1.54), rectal (1.30, 1.10-1.54; 1.50, 1.13-1.99), and liver (2.20, 1.74-2.78; 1.79, 1.09-2.93) cancers in men and women. In men, diabetes was positively associated with pancreatic (1.46, 1.23-1.74) and urinary (1.10, 1.01-1.20) cancers. In women, diabetes was positively associated with stomach (1.61, 1.05-2.47), anal (2.50, 1.21-5.18), and endometrial (1.29, 1.10-1.52) cancers. Discussion: Diabetes was associated with lower risk of incident prostate cancer in men, but with higher risk for a number of other cancers in both sexes. Diabetes was also associated with higher risk for cancer mortality. Our results suggest that preventing or controlling diabetes may contribute to decreased cancer incidence and mortality.

NCI-DCEG
Menashe, Idan
Visiting Fellow
Epidemiology/Biostatistics - Etiology, Risk, and Prevention
Pathway-based analysis of bladder cancer genome-wide association study suggests involvement of cellular detoxification processes.
Bladder cancer genome-wide association studies (GWAS) have conclusively identified ten susceptibility loci however estimates suggest several more remain unknown. To identify additional susceptibility loci, and gain further insight into the biological mechanisms underlying bladder cancer etiology, we applied pathway analysis to a bladder cancer GWAS containing 3532 cases and 5120 controls of European descent from five studies. We collected 1399 pathways from five publically available resources (Biocarta, Kegg, NCI-PID, HumanCyc, and Reactome), and constructed another 23 candidate pathways relevant to bladder cancer risk factors. SNPs were mapped to genes if they were located within 20kb 5’ upstream

and 10kb 3' downstream of the gene's coding region. Overall, 1422 pathways, 5647 genes and ~90,000 SNPs were included in our study. Logistic regression models adjusting for age, sex, study, DNA source, and smoking status was used to assess the marginal trend effect of SNPs on bladder cancer risk. The I2 statistic was used to identify SNPs with heterogeneous effects across the five study populations. The most significant SNP in each gene was used in the pathway analysis. Two complementary pathway-based methods (gene-set enrichment analysis [GSEA], and adapted rank-truncated product [ARTP]) were used to assess the enrichment of association signals within each pathway. Eighteen pathways were detected by either GSEA or ARTP at the P<0.01 level. To avoid false positives, we removed SNPs displaying heterogeneous effects (I2; P < 0.2) from genes in the top pathways. Post heterogeneity analysis, the enrichment signals remained significant in seven pathways delineating three metabolic processes (Aromatic amine metabolism, Nicotinamide adenine dinucleotide metabolisms, Clathring-mediated vesicles), and one cell cycle (Mitotic Metaphase/Anaphase transition) pathway. Identification of the aromatic amine metabolism pathway supports the ability of our method to identify pathways with established relevance to bladder cancer susceptibility. Interestingly, all three identified metabolic pathways play a role in cellular detoxification which is a known anti-cancer mechanism. These results suggest involvement of metabolic detoxification in bladder cancer susceptibility. Further studies are needed to confirm our findings, and examine the specific role of these pathways in bladder carcinogenesis.

NCI-DCEG
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Epidemiology/Biostatistics - Etiology, Risk, and Prevention
Estrogen plus progestin menopausal hormone use: A safe regimen of use with respect to endometrial cancer risk?
BACKGROUND: Given extensive evidence linking unopposed estrogens to substantial increases in endometrial cancer risk, it has become standard practice to prescribe estrogens in conjunction with a progestin, particularly for women with intact uteri. It is widely assumed that this regimen is safe, but few studies have been able to thoroughly evaluate risk according to regimen (sequential vs. continuous use of progestins) or length of use. Further, associations stratified by body mass index (BMI), a major endometrial cancer risk factor as well as an important modifier of unopposed estrogen risks, remain unclear. METHODS: We evaluated the relationship of menopausal hormone use and incident endometrial cancer (n=885) in a cohort of 68,479 postmenopausal women enrolled in the NIH-AARP Diet and Health Study. The cohort was followed from completion of a questionnaire administered between1996-1997 through 2006. Relative risks (RR) and 95% confidence intervals (CI) were estimated using Cox regression. RESULTS: Among 19,131 women (27.9% of analytic population) who reported exclusive use of estrogen plus progestin regimens, 176 developed endometrial cancer [RR 0.88; 95% CI 0.74, 1.06]. Compared with women who never used hormones, long-duration (>= 10 years) estrogen plus progestin use was positively associated with endometrial cancer [RR 1.37; 95% CI 1.07-1.75]. This risk was largely driven by higher risks related to current [RR 1.32; 95% CI 1.01, 1.72] or long-duration [RR 1.38; 95% CI 1.05, 1.81] sequential (<15 days/month) progestin use. We further observed that normal weight women (BMI < 25 kg/m2) comprised the only BMI subgroup in whom estrogen plus progestin use was associated with increased risk [RR 1.78; 95% CI 1.28, 2.47]. The positive association was apparent among estrogen plus progestin users who reported current [RR 1.94; 95% CI 1.38, 2.71], long-duration [RR 2.92; 95% CI 1.96, 4.34] or sequential regimen [RR 2.53; 95% CI 1.71, 3.74] usage. CONCLUSION: Our findings showed that estrogen plus progestin therapy may not be totally safe with respect to endometrial cancer risk, particularly when long-durations of sequential progestins are involved. Similar to previous findings for unopposed estrogens, normal weight women, with lower

endogenous estrogen levels, were those most affected by estrogen plus progestin use--providing additional evidence that menopausal hormones and obesity increase endometrial cancer occurrence through common etiologic pathways.

NCI-DCEG
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Epidemiology/Biostatistics - Prognosis and Response Predictions
Risk of lung cancer associated with domestic use of different types of coal in Xuanwei, China
About half of the world's population uses unprocessed biomass fuels and coal for cooking and heating. Recently, the International Agency for Research on Cancer (IARC) classified emissions of indoor combustion of coal as carcinogenic to humans. However, variation in cancer risk by coal subtype still needs to be addressed. The present study evaluated the different effect of lifelong use of two different types of coal, "smoky coal" (bituminous) and "smokeless coal" (anthracite), on lung cancer mortality among residents in Xuanwei, China. Rates of lung cancer in this area are among the highest in China, and are associated with heavy exposure to indoor coal emissions. A cohort of 37,753 farmers, born from 1917 through 1951, was followed up from 1976 through 1996. Association of type of coal used with lung cancer mortality was analyzed with nonparametric survival analysis and multivariable Cox models adjusting for demographic factors, smoking status, family history of lung cancer, occupational history, and type and number of stoves used in the household. The relationship between average number of hours per day spent indoor and risk of lung cancer was investigated allowing for possible non-linear effects by including natural cubic splines in the Cox models. During the study period, there were 9,845 deaths from any cause and 2,445 from lung cancer. After adjusting for the presence of competing risks of death, the probabilities of death from lung cancer before 80 years of age for men and women using smoky coal were 18% and 20%, respectively, compared with less than 0.5% among smokeless coal users of both sexes. Before age 60, lung cancer alone accounted for about 40% of deaths among smoky coal users. Compared with smokeless coal, use of smoky coal was associated with a substantial increase in risk of lung cancer (men: Hazard Ratio [HR], 37; 95% Confidence Interval [CI], 22-51; women: HR, 121; 95% CI, 50-293). A monotonic increase in risk of lung cancer with longer time spent indoor was observed among smoky coal users of both sexes, while no clear trend was observed among smokeless coal users. These findings suggest that the carcinogenic potential of coal combustion products exhibit substantial variation by specific coal type. Additional studies are warranted to better characterize the carcinogenic potential among coal subtypes. Use of less carcinogenic types of coal can translate into a substantial reduction of lung cancer risk.

NCI-DCEG
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Epidemiology/Biostatistics - Prognosis and Response Predictions
Immunogenetics and risk of mortality after lung cancer diagnosis
Background: Lung cancer mortality is the leading cause of cancer death worldwide, with the highest rates in high-income countries. Smoking accounts for the majority of lung cancer deaths, but genetic predisposition is thought to play a role as evidenced by the significant fraction of non-smokers dying from lung cancer. It is known that lung cancer mortality is increased in patients with chronic obstructive pulmonary disease, an inflammatory disease of the lung, and that smoking induces inflammatory alterations in the lung microenvironment. These findings suggest inflammatory related pathways as potential candidates for genetic predisposition to poor survival. Methods: Using data from a population-based retrospective study, we conducted a analysis of the common variation in inflammatory related

genes and risk of mortality after lung cancer diagnosis. We genotyped a total of 19,082 single nucleotide polymorphisms (SNPs) in 970 genes. Hazard ratios (HR) and corresponding 95% confidence intervals (CI) were computed using Cox regression, assuming a log-additive model for the genotype. All analyses were adjusted for age at the time of diagnosis, stage of the disease, smoking status, number of pack-years and sex. Results: A total of 2,100 lung cancer cases were included in the analysis with a median of 1.27 years of follow-up (interquartile range: 0.56-3.44 years). A SNP in the flanking region of the HDAC2 gene, a gene implicated in transcriptional regulation and cell cycle progression, was associated with all cause mortality following lung cancer diagnosis (P<10-5). Specifically, the T allele of SNP rs2281674 decreased the risk of mortality by 19% (HR=0.81; 95% CI: 0.74-0.89) as well as the risk of lung cancer specific mortality by 20% (HR=0.80, 95% CI: 0.72-0.88). Moreover, in a gene analysis, HDAC2 was also associated with survival (P<0.0005). Stratified analysis suggested that the C allele of SNP rs11721827 in the toll-like receptor 3 (TLR3) gene, which plays a critical role in the activation of the innate immunity, was found to increase the risk of mortality among never smokers (HR=3.20, 95% CI: 2.03-5.04). Conclusions: In this first large study of the genetic variation in inflammatory genes and risk of lung cancer mortality, we found evidence that polymorphisms in HDAC2 and TLR3 might contribute to survival after lung cancer diagnosis. These results could lead to future therapeutic strategies.

NHGRI
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Epidemiology/Biostatistics - Prognosis and Response Predictions
Targeted deep resequencing identifies coding variants in the PEAR1 gene that play a role in platelet aggregation in individuals at high risk for coronary artery disease
Background: Aggregation of activated platelets on atherosclerotic plaques is a critical step in the initiation of thromboses of the arterial system, which results in coronary artery disease (CAD). The Genetic Study of Aspirin Responsiveness (GeneSTAR) is a family-based study, designed to examine genetic determinants of platelet aggregation in African American (AA) and European American (EA) healthy subjects with a family history of CAD. Platelet aggregation is heritable, and a previous genomewide association study (GWAS) detected strong association with a common genetic variant in intron 1 of the platelet endothelial aggregation receptor1 (PEAR1) gene in AA and EA GeneSTAR subjects. Build upon these results, we exploit sequencing approach designed to identify additional functional genetic variants in PEAR1 that may also play a role in platelet aggregation. Methods: 104 subjects were selected based on the hyper (AA: n=24; EA n=26) and hypo (AA: n=27; EA n=27) aggregation across three different platelet agonists (Collagen, Epinephrine, and ADP). A 0.3Mb target including PEAR1 was Sanger sequenced at deCODE Genetics. Variants were annotated for function and novelty. Single-variant tests for association (variant presence/absence vs. hyper/hypo aggregation) were performed using Fisher's Exact Test. Collapsed multi-variant tests for association were performed using the Combined Multivariate Collapsing and the Weighted Sum methods. For these tests, variants were collapsed based on: 1) minor allele frequency (MAF) and 2) predicted function. Analyses were performed separately in AAs and EAs. Results: 235 variants were identified, and 104 were novel. Sixteen missense variants were observed, ten of which were novel. More rare variants (MAF < 5%) were noted in AAs compared to EAs (108 vs. 45). The common intronic GWAS-identified variant (rs12041331) has the most significant association signal in the AA sample (p=7.9E-5); no additional association was seen for exonic variants. On the other hand, collapsed multi-variant tests suggest that exonic variants play a more significant role in the EA sample (p = 0.01 for the collapsed coding variants compared to p = 0.05 for rs12041331). Conclusion: Genetic variants in the PEAR1 are determinants of an individual's platelet aggregation response. We confirm that a common intronic variant has the strongest association in AAs, and show that additional exonic variants play a role in platelet aggregation, particularly in EAs.

NCI-DCEG
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Epidemiology/Biostatistics - Prognosis and Response Predictions
Variation in the risk of radiation-related breast cancer by estrogen receptor expression and histology
Exposure to ionizing radiation is an established risk factor for breast cancer. However, breast cancer is not one but several diseases, varying by histology and hormone receptor expression. While reproductive and hormonal risk factors for breast cancer are associated with certain molecular subtypes and not others, it is not known whether the risk of radiation-related breast cancer varies by molecular subtype. Few studies have the necessary data to examine this question because they either lack information on radiation exposure or hormone receptor status. The Surveillance Epidemiology and End Results database provides a unique opportunity to investigate the relationship between radiation and breast cancer by molecular subtype in breast cancer survivors who were treated with radiotherapy and for whom long-term follow-up on subsequent contralateral breast cancer diagnosis with tumor specifications are available. We characterized the risk of contralateral breast cancer (CBC) related to radiotherapy by histology and estrogen receptor (ER) status in five-year survivors using Poisson regression models adjusted for attained age and calendar year, age at and year of treatment, and disease stage. Overall 205,316 female breast cancer survivors were followed for an average of 10 years from 1973 until 2007, during which time 6924 women developed a subsequent primary invasive breast cancer in the contralateral breast. Information on ER status is available from 1990 onwards. Of the 3546 cases with this information, 2597 were ER+ and 949 were ER-. The overall risk (and 95% confidence interval (CI)) of CBC related to radiotherapy was 1.11 (1.05, 1.16). Relative risks (and 95% CI) were 1.09 (1.01, 1.18) for ER+ CBC and 1.16 (1.02, 1.31) for ER- CBC. Among women treated before age 35 (n=6305), the most radiosensitive subgroup, radiation-related risk of CBC was elevated for ER- tumors (RR=1.37, 95% CI: 0.96, 1.96) but not for ER+ tumors (RR=0.87, 95% CI: 0.48, 1.39) (p=0.11). Risks were elevated to a similar degree among women treated after age 35 (p>0.50). There was no heterogeneity in risk according to histology of the CBC (p>0.50), but case numbers were small for subtypes other than ductal and lobular carcinomas. In this large study with long-term follow-up, we found that radiation may be more strongly related to ER- tumor types. While further studies are needed to confirm this finding, it may provide clues to the mechanism of radiation-related breast cancer risk.

NIDDK
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Epigenetics
Distinct roles of GCN5/PCAF-mediated H3K9ac and CBP/p300-mediated H3K18/27ac in nuclear receptor transactivation
Histone acetylation generally correlates with gene activation, although it remains to be established whether changes in histone acetylations are a cause or a consequence of the increased transcription. The two pairs of mammalian histone acetyltransferases (HATs), GCN5/PCAF and CBP/p300, have been shown to function as coactivators for hundreds of transcription factors including nuclear receptors. However, the substrate and site specificities of GCN5/PCAF and CBP/p300 in vivo, as well as the roles of GCN5/PCAF- and CBP/p300-mediated histone acetylations in regulating ligand-induced nuclear receptor target gene expression, remain largely unclear. Here we show in mouse embryonic fibroblasts (MEFs), deletion of GCN5/PCAF specifically eliminates acetylation on K9 of histone H3 (H3K9Ac) while deletion of CBP/p300 selectively reduces acetylation on K18 and K27 of histone H3 (H3K18/27Ac). Treating MEFs with a specific ligand for nuclear receptor PPARdelta induces sequential increases of H3K18/27Ac and

H3K9Ac on the promoter of PPARdelta target gene Angptl4, which correlates with a robust ligand-induced Angptl4 expression. Inhibiting transcription elongation by DRB blocks ligand-induced H3K9Ac but not ligand-induced H3K18/27Ac on Angptl4 promoter. Finally, we show CBP/p300 are essential, while GCN5/PCAF and GCN5/PCAF-mediated H3K9Ac are dispensable, for ligand-induced PPARdelta target gene expression in MEFs. These results highlight the substrate and site specificities of HATs in cells, and suggest that GCN5/PCAF- and CBP/p300-mediated histone acetylations play distinct roles in regulating ligand-induced nuclear receptor target gene expression.

NCI-CCR
wang, chunxi
Visiting Fellow
Epigenetics
EZH2 mediates epigenetic silencing of Neuroblastoma tumor suppressor genes CASZ1, CLU, RUNX3 and NGFR
Neuroblastoma (NB) is the most common solid extracranial pediatric tumor arising from the neural crest. Patients with poor prognoses have undifferentiated, stroma-poor tumors in contrast to the differentiated, stroma-rich tumors of patients with good prognoses. The Polycomb-group protein, EZH2, is a histone methytransferase that regulates differentiation and is dysregulated in many types of tumors. Higher EZH2 expression is found in undifferentiated, stroma-poor NB tumors compared to the more differentiated tumors (p=4.12x10-8), Microarray data from 88 clinical NB tumor samples shows high EZH2 expression is associated with poor prognosis (p=4.2x10-4). We hypothesize that high EZH2 expression maintains the undifferentiated state common to poor prognosis NB by repressing tumor suppressors. To test this we evaluated the regulation of a newly identified NB tumor suppressor, CASZ1, whose restoration inhibits NB growth and induces differentiation. Low CASZ1 expression is significantly associated with poor prognosis in NB (p=0.0011) and there is a statistically significant inverse correlation between EZH2 and CASZ1 expression (p=2.55x10-5). Decreasing EZH2 expression in NB using EZH2 shRNA or a pharmacological EZH2 inhibitor, 3-deazaneplanocin A (DZNep), results in increased CASZ1 expression. Furthermore, CASZ1 mRNA expression is 3-fold higher in EZH2-/- mouse embryonic fibroblasts (MEFs) compared to EZH2+/+ MEFs. HDAC inhibitors (HDACi) increase CASZ1 expression in four NB cell lines (SK-N-AS, SH-SY5Y, SMS-KCNR and NGP), which is associated with decreased protein expression of PRC2 components, EZH2 and SUZ12. Chromatin Immunoprecipitation (ChIP) indicates enriched H3K27me3 and PRC2 components binding to the CASZ1 transcriptional start site (TSS) under steady-state conditions. PRC2 component binding and H3K27me3 enrichment are decreased after HDACi treatment, while RNA polymerase II binding and H3K4me3 enrichment increase, consistent with the increased CASZ1 expression. Using similar strategies we found also find that other reported NB tumor suppressors, CLU, NGFR and RUNX3 are also directly repressed by EZH2-mediated H3K27me3 repression in NB cells. Decreasing EZH2 expression using shRNA or DZNep inhibits NB cell growth and induces neurite extensions. Our study implicates that aberrant EZH2 mediates epigenetic gene silencing of tumor suppressors as a mechanism that may contribute to NB tumorgenesis and its undifferentiated phenotype.

NIDDK
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Visiting Fellow
Epigenetics
Histone H3K27 demethylase UTX is required for mesoderm differentiation of embryonic stem cells
Embryonic stem (ES) cells are capable of differentiating into all three germ layers endoderm, ectoderm and mesoderm in vitro, which mimics early embryonic development in vivo. ES cell differentiation is

controlled by complex interplay between extracellular signaling and dynamics of core developmental regulators. Research on ES cells provides insight into molecular mechanisms underlying mammalian development. Recent studies indicate that the histone H3K27 methyltransferase PRC2 (Polycomb Repressive Complex 2) represses a large number of developmental regulators and is critical for proper differentiation of ES cells. To investigate the role of dynamic change of histone H3K27 methylation in ES cell differentiation, we have deleted the gene encoding histone H3K27 demethylase UTX in mouse ES cells. Deletion of UTX does not affect proliferation, colony formation ability or differentiation potential of ES cells. UTX is also dispensable for retinoic acid-induced ES cell differentiation towards neuronal precursor cells. However, when induced to differentiate into embryoid bodies, UTX knockout (KO) ES cells show severe morphological defects and fail to express mesoderm markers including Brachyury, a transcriptional factor essential for mesoderm differentiation. In contrast, expression of endoderm markers is largely unaffected. Chromatin immunoprecipitation (ChIP) assay indicates that UTX directly and selectively binds to Brachyury promoter in ES cells. Deletion of UTX impairs Wnt/beta-catenin signaling-induced Brachyury expression and leads to increased H3K27me3 and PRC2 levels as well as decreased H3K27ac and H3K4me3 levels on Brachyury promoter. Further, UTX physically interacts with transcription factors LEF1/TCF7, which act downstream of the Wnt/beta-catenin signaling pathway to control Brachyury induction. Taken together, these results indicate that histone H3K27 demethylase UTX is selectively required for ES cell differentiation towards mesoderm, and suggest that UTX antagonizes H3K27 methyltransferase PRC2 to promote Brachyury expression and mesoderm differentiation in ES cells.

NIDDK
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Epigenetics
Epigenetic Regulation of Adipogenesis
Background: Obesity and type-II diabetes are two major health problems in USA. Obesity is the most important risk factor for type-II diabetes. Understanding how adipogenesis (generation of adipose tissue) is regulated may provide new ways to treat obesity and type-II diabetes. This study focuses on epigenetic regulation of adipogenesis using both cellular and mouse models. Objective: The principal adipogenic transcription factors PPARgamma and C/EBPalpha cooperate to promote adipogenesis. In contrast, Wnt/ß-catenin signaling inhibits adipogenesis. We are investigating how epigenetic mechanisms regulate these positive and negative master regulators of adipogenesis. Results: Di-methylation on histone 3 lysine 9 (H3K9me2) and tri-methylation on histone 3 lysine 27 (H3K27me3) are two major repressive marks for gene expression. Here we show these two repressive epigenetic marks play opposite roles in adipogenesis. The H3K9 methyltransferase G9a represses adipogenesis. G9a directly represses the expression of adipogenesis master regulator PPARgamma before and during adipogenesis by adding H3K9me2 on its promoter. Meanwhile, G9a is required for expression of adipogenesis inhibitor Wnt10a in a manner that is independent of its methyltransferase activity. Deletion of G9a increases PPARgamma expression and decreases Wnt10a expression, which synergistically promote adipogenesis. Consistent with these results, adipose tissue-specific deletion of G9a in mice significantly increases adipocyte gene expression, fat accumulation, as well as fat tissue and body weights. In contrast, H3K27 methyltransferase Ezh2 and its enzyme activity are required for adipogenesis. Ezh2 directly represses Wnt genes before and during adipogenesis. Deletion of Ezh2 eliminates H3K27me3 on Wnt promoters and de-represses Wnt expression, which leads to activation of Wnt/beta-catenin signaling and inhibition of adipogenesis. The adipogenesis defects in Ezh2-/- cells can be rescued by expression of either adipogenic transcription factors PPARgamma, C/EBPalpha, inhibitors of Wnt/beta-catenin signaling, or the wild-type, but not the enzymatically inactive mutant, Ezh2.

Conclusions: Taken together, these results provide an initial view of epigenetic regulation of adipogenesis. Our data suggest that by regulating the expression of both positive and negative master regulators of adipogenesis, methylations on histone H3K9 and K27 play crucial but opposite roles in adipogenesis.

NCI-CCR
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Postdoctoral Fellow
Gene Expression
Baseline chromatin accessibility is an important determinant for GR binding patterns in murine liver tissue
Glucocorticoids are the major therapy used for management of auto-inflammatory diseases. Glucocorticoid receptor (GR) dysregulation is implicated in the pathogenesis of stress-related disorders and linked to chronic inflammation associated with the metabolic syndrome. How the glucocorticoid receptor targets specific regions of the genome to elicit physiological responses is a largely unanswered question. Recently, we have mapped genome wide GR binding patterns in multiple cell lines by ChIP-seq. Coupling ChIP-Seq with DHS-Seq, an assay that identifies open regions of chromatin, we have shown that GR occupancy is highly cell-type specific and is co-related with the baseline (i.e. prior to hormone treatment) chromatin landscapes found in each cell line. These studies have begun to address mechanisms regulating GR occupancy of the genome in cell lines, but little is known about the mechanisms controlling genome accessibility of GR in tissues. Here, we attempt to uncover some of the molecular mechanisms that regulate GR binding to target sites in the murine liver genome. To study the effects of glucocorticoids in liver tissue, we disrupted GR activity in all tissues of C57BL/6 mice by surgical removal of the adrenal glands, the exclusive organ for GR ligand (corticosteroid) synthesis. Three day post surgery, adrenalectomized mice were injected with the synthetic GR agonist, dexamethasone or vehicle for one hour. ChIP-seq analysis for GR uncovered ~10,000 highly confident, reproducible GR binding sites in liver. We also optimized the DNAse-seq procedure to be used for liver tissue. Remarkably, we found that the majority of GR binding sites fall into the baseline accessible chromatin compartment, just as is observed in cell lines. De novo DNA motif analysis suggests that C/EBP elements occupy the accessible chromatin compartment prior to GR binding. Comparison with a C/EBP binding profile from liver shows that 80% of GR binding in the accessible chromatin compartment overlaps with C/EBP occupancy, indicating that C/EBP might regulate genome accessibility of GR in liver. In order to uncover the functional role of C/EBP in liver we have generated a mouse strain that expresses a highly specific dominant negative form of C/EBP under a liver specific conditional expression system. The functional role of C/EBP in GR binding, chromatin accessibility and genome organization will be discussed in the context of GR function in the murine liver.

NIDCR
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Gene Expression
Viral Gene Transfer to Developing Mouse Salivary Glands
Branching morphogenesis is essential for the formation of salivary gland, kidney, lung, pancreas, and many other organs during development, but the mechanisms of this crucial process are not adequately understood. Microarray and serial analysis of gene expression (SAGE) have been powerful approaches to identify candidate genes that could potentially regulate branching morphogenesis. However, functional validation of these genes has been severely hampered by the absence of efficient techniques to genetically manipulate cells within embryonic mammalian organs. Using ex vivo cultured embryonic

mouse salivary glands as a model organ system to study branching morphogenesis, we have identified new vectors for viral gene transfer with high efficiency and cell-type specificity to developing salivary glands. We screened adenovirus, lentivirus and 20 types of adeno-associated viruses (AAV) for their ability to transduce early stage (embryonic day 12 or 13) salivary glands. Green fluorescent protein was engineered into each of these viruses to facilitate calculations of transduction efficiency. The panel of AAVs tested included not only different serotypes but also AAV capsid mutants. We found that adenovirus transduced isolated salivary epithelial rudiments, but in intact glands its access to epithelium was blocked by the outer mesenchymal cells. Lentivirus did not transduce either cell type in this model. We identified two AAVs, self-complementary AAV2 (scAAV2) and bovine AAV (BAAV), that are highly selective at transducing salivary epithelial and mesenchymal cell populations, respectively. Transduction of salivary epithelia with scAAV2 that expresses fibroblast growth factor 7 (FGF7) supported gland survival and enhanced branching morphogenesis. Microinjection of adenovirus that expresses a novel regulatory gene called Btbd7 (BTB domain containing 7 gene) in the salivary epithelium also resulted in a significant increase in branching morphogenesis, compared to glands microinjected with control virus. Our findings represent the first successful selective targeting to epithelial versus mesenchymal cells in an organ undergoing branching morphogenesis, and the first use of gene transfer to analyze roles of growth factors and novel regulatory genes in this process of mammalian organ morphogenesis.

NCI-CCR
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Postdoctoral Fellow
Gene Expression
Gene and microRNA expression signatures reveal unique molecular targets for therapeutic intervention of etoposide resistant breast cancer
Breast cancer is the second leading cause of cancer death for women in the U.S and etoposide is the drug of choice for advanced stage breast cancer, however, resistance to this drug is a major roadblock in the effective treatment of breast cancer. In order to develop targeted therapeutic strategies to combat drug resistance it is essential to understand the basic molecular mechanisms through which cancer cells control sensitivity to chemotherapeutics. We have carried out extensive studies using a number of tools including microarrays, microRNA arrays, siRNA and miRNA mediated gene silencing to identify novel candidate genes and microRNAs involved in etoposide resistance. Using a microarray approach we have generated a gene expression signature for etoposide resistance in MCF7VP (etoposide resistant) breast cancer cells. We utilized U133 plus 2 high-density oligonucleotide microarrays along with ABC transporter microfluidic arrays and validated the gene expression data with qRTPCR. Drug sensitive MCF7 cells were used as controls. We discovered differential expression of over 5000 genes (fold change > 1.5, P value < 0.05). The resistance mechanisms identified include upregulation of ABC transporter genes (ABCC1 and ABCC6), downregulation of the drug target (Topoisomerase II) and downregulation of apoptotic pathway genes (CASP7, DIABLO). Several key transcription factors such as RUNX2, ETS1, SOX9 and SMAD3 were upregulated in MCF7VP cells suggesting that these genes could be explored as therapeutic targets. Targeted RUNX2 knockdown in the resistant cells using siRNA increased sensitivity to etoposide and also upregulated expression of pro-apoptotic genes indicating that RUNX2 could be a molecular target against etoposide resistance. We additionally observed a distinct miRNA signature for etoposide resistant cells suggesting that miRNA may play a major functional role in the regulation of drug resistance. Hsa-miR-218 was downregulated in the MCF7VP cell line. This miRNA is predicted to target ABCC6. We found that transfection of a miR-218 mimic could downregulate the expression of the efflux pump ABCC6 by almost 65% in drug resistant cells and may be explored as a potential therapeutic agent to circumvent drug resistance. This is the first extensive study in which novel molecular

targets/biomarkers for etoposide resistance have been identified which can be further developed as clinical therapies to overcome etoposide resistance.

NIMH
Newburn, Erin
Postdoctoral Fellow
Gene Expression
Disease-specific Changes in the Expression of PSD-95 Variants in Patients with Schizophrenia and Affective Disorders
Disturbances in glutamatergic neurotransmission and synaptic changes in schizophrenia have led to the investigation of postsynaptic density-related molecules as potential pathological factors. PSD-95, a scaffolding adaptor protein encoded by the DLG4 gene, serves a critical role in complexing multiple proteins into a functional unit for relaying cellular signaling cues. In the present study, we examined the expression of mRNA splice variants of PSD-95 (PSD95-a, -ß, and truncated) in the dorsolateral prefrontal cortex (DLPFC) and hippocampus in a large developmental cohort (N=326) of normal non-psychiatric individuals from fetal to 83 years of age and in a second, diagnostic group of patients with schizophrenia (N=176) and affective disorders (Bipolar, N=61; Major Depression, N=138). PSD95 variants showed unique neurodevelopmental expression patterns across the human lifespan. While PSD95-a was expressed at low levels during the fetal period, followed by a gradual increase in expression and a peak at young adulthood, and ß showed a relatively flat profile throughout life, the truncated variant was very highly expressed in the fetal stage and declined rapidly thereafter suggesting its importance in early development. Abnormalities in PSD95 splice variants were disease and region-specific. While no alterations were detected in the predominant isoform (PSD95-a), patients with schizophrenia had significantly lower PSD95ß transcript levels in the DLPFC (p=3.4x10-5). In contrast, PSD95ß was higher in patients with major depressive disorder (p=5.8x10-7). The truncated transcript was significantly decreased in both regions in patients with schizophrenia (p<0.018), decreased in the DLPFC (p=1.9x10-6) of patients with bipolar disorder, yet, increased in the hippocampus (p=6x10-4) of bipolar patients. Furthermore, several SNPS (single nucleotide polymorphisms) in the DLG4 gene showed an association with transcript expression. SNP (rs929229), which has been previously associated with schizophrenia and lower cognitive performance, predicted expression of PSD95ß (p=0.04). Determining specific splice variant transcript expression may help understand molecular perturbations of glutamatergic intracellular signaling in the prefrontal cortex and hippocampus of psychiatric patients. Moreover, the associations between numerous DLG4 SNPs and mRNA transcript expression levels suggest a genetic contribution to the neuropathology of these disorders.

NLM
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Postdoctoral Fellow
Gene Expression
Genome-scale analysis of promoter melting in eukaryotic gene transcription
Eukaryotic gene transcription is a highly regulated process and can be divided into several phases, including RNA polymerase II (Pol II) recruitment, preinitiation complex assembly, open complex formation, promoter escape, elongation and termination. Pol II recruitment to promoter was believed to be the predominant step at which gene expression is regulated. However, recent genome-wide studies demonstrate that a large number of genes is also regulated after transcription initiation through pausing of Pol II proximal to promoters. We demonstrated that the transition from closed to open Pol II complex formation, which is marked by the appearance of a “bubble” of melted DNA, is a third key regulatory step of gene expression in mammalian cells. To study this event genome-wide, we developed a new

experimental method that combined chemical and enzymatic techniques with high-throughput sequencing (ssDNA-Seq) to map the position and extent of single-stranded DNA (ssDNA) associated with transcription. The method specifically targeted single-stranded DNA regions by selective oxidation of unpaired bases by potassium permanganate and it allowed us to visualize conformational changes in canonical B-DNA structure in vivo. Using this new genome-wide approach, we built a comprehensive map of single-stranded DNA associated with transcription in mouse activated and resting B cells. In activated B cells, ssDNA-Seq sites reflecting promoter melting generally corresponded to active promoter regions, as evidenced by Pol II recruitment and gene expression. The promoter melting was directly modulated by the rate of Pol II promoter escape and the presence of non-B DNA conformations. Moreover, the formation of a stable transcription bubble was rate-limiting for a subset of genes. Especially, in resting B cells that display only basal gene expression, polymerase complexes were mostly found in a closed form. Therefore, mouse B cells control gene transcription mostly by globally limiting open complex formation in resting cells or by polymerase pausing proximal to promoters upon activation. In summary, we identified the promoter melting as a third key regulatory step in eukaryotic gene transcription. Our new approach was crucial to better understand the dynamics of promoter melting. It can be used in other cell types and organisms to help establish the further role of DNA structure in gene regulation.

NCI-CCR
Zhang, Geng
Research Fellow
Gene Expression
DPEP1 and TPX2 as Independent Predictors of Cancer-Specific Mortality in Pancreatic Ductal Adenocarcinoma
Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal cancers worldwide with a median survival of ~6 months. Because of the current lack of early detection strategies, > 80% of patients present with advanced disease at diagnosis. The overall 5-year survival for PDAC patients is only 3-5%. The dismal prognosis is attributed to early metastatic spread and resistance to chemotherapy and radiotherapy. Therefore, better biomarkers and novel therapeutic targets are desperately needed to improve the survival rate of patients with PDAC. In current study, we aimed to identify biologically relevant genes with prognostic and therapeutic significance in PDAC. We compared the microarray gene-expression profiles of 45 matching pairs of pancreatic tumor and adjacent non-tumor tissues. 53 Genes that were differentially expressed and associated with survival, were evaluated using quantitative RT-PCR in the test cohort (N=45) and further validated in an independent validation cohort (N=27). Our analysis revealed that a lower expression of DPEP1 (dipeptidase 1) and a higher expression of TPX2 (targeting protein for Xklp2) in tumors are associated with poor survival in both test and validation cohorts (P<0.05, Kaplan-Meier log-rank test). DPEP1 (HR, 0.51; 95% CI, 0.27-0.96) and TPX2 (HR, 2.31; 95% CI, 1.18-4.52) expressions were independently associated with cancer-specific mortality when adjusted for tumor stage and resection margin status in both univariate and multivariate analyses. In tumor tissues, DPEP1 expression was decreased (T: N ratio ~0.1, P<0.01) and TPX2 expression was increased (T: N ratio ~2.14, P<0.001), compared to non-tumor tissues in both cohorts. Furthermore, using mouse model and cell lines, we demonstrated that mutant KRAS and growth factors such as EGF can decrease DPEP1 and increase TPX2 expression through MAPK pathway, shedding light on the molecular mechanism of DPEP1 and TPX2 alterations in tumorigenesis and progression. We found MAPK specific inhibitor U0126 significantly enhanced DPEP1 and suppressed TPX2 level, indicating a novel rationale for targeting oncogenic RAS/MAPK pathway in the treatment of PDCA. In conclusion, our data provide the first evidence that DPEP1 and TPX2 are prognostic biomarkers, independent of resection

margin status and other clinical covariates in multiple cohorts of PDAC, and may be useful in identifying high-risk patients in order to guide treatment decisions.

NIDCR
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Research Fellow
Gene Therapy
Salivary Glands are Useful Target Organs for Gene Therapy Treatment of Diabetes Mellitus (DM)
Using several animal models, our group has previously demonstrated salivary glands (SGs) are a safe and relatively easy gene transfer target to treat monoendocrinopathies. Here, an adenoviral vector encoding the human proinsulin (hproins) B10 variant (Ad-proins-B10) was generated and tested. This variant differs from wild type hproins by an Histidine to Aspartic acid substitution in the B-chain and rapidly exits pancreatic beta-cells via the constitutive secretory pathway; the pathway which, in SGs, leads to the circulation. Experiments using Ad-proins-B10 were performed to determine the route of hproins secretion in SGs, and to test the ability of SG-generated hproins to lower blood glucose in mice. Twenty-four hours after in vitro transduction with Ad-proins-B10, HEK 293 cells, A5 rat SG epithelial cells, and NIT-1 mouse pancreatic beta-cells all secreted hproins into culture media. An in vitro assay based on phosphorylation of Akt at Ser473 following activation of the insulin receptor, showed that the hproins present in conditioned media from Ad-proins-B10-transduced 293 cells was bioactive. Ad-proins-B10 was delivered in increasing doses to the SGs of male Balb/c mice by retroductal infusion. A dose-dependent increase in serum hproins was seen at 24h and was associated with a decrease in blood glucose levels at this time. Very low levels of hproins were detected in the lysates of Ad-proins-B10-transduced SGs and hproins was undetectable in the saliva of mice at the end of the experiment. Intravenous delivery of Ad-proins-B10 to the liver induced similar levels of circulating hproins at 24h. Taken together, these results indicate that transgenic hproins-B10 expressed by the SGs is primarily secreted to the circulation at high levels. Experiments using the beta-cell-specific cytotoxic drug Alloxan (ALX) were performed to test the value of Ad-proins-B10 delivery to SGs as a potential treatment for Type I DM. Briefly, all mice were fasted 16h prior to I.P. injection of ALX, treated with either Ad-proins-B10 or Ad-control and serum glucose and hproins levels were monitored. Consistent with previous experiments, serum hproins was significantly elevated in Ad-proins-B10-treated diabetic mice at 24h post-treatment. Furthermore, these mice had blood glucose levels reduced nearly 2-fold when compared to Ad-control-treated diabetic mice. These results demonstrate the potential of hproins-B10 gene delivery to SGs as a therapy for Type I DM.

FDA/CBER
Xu, Zhili
Postdoctoral Fellow
Gene Therapy
Coagulation factor X shields adenovirus vectors from natural antibodies and complement: impact on liver transduction
Adenovirus vector type 5 (Ad) is the most widely-used vector in clinical gene therapy trials, due to its advantages of high capacity for therapeutic genes, high transduction efficiency and ease of manufacture. However wider clinical success is hampered by Ad vector's one major shortcoming: most systemically-administered Ad virions are sequestered into the liver, limiting their ability to reach other therapeutic targets such as metastatic tumors. The mechanisms underlying this have not been clear. Recently two papers in top journals showed that the major adenovirus capsid protein (hexon) has a high-affinity binding site for a plasma protein, coagulation Factor X (FX), and that FX plays a central role in the ability of Ad vectors to transduce the liver. Ad becomes unable to transduce mouse liver when FX

is blocked or depleted, or when hexon is mutated at the FX-binding site. The papers conclude that FX acts a bridge receptor to facilitate hepatocyte transduction, with the hypothesis that one end of FX binds to the Ad capsid, and the other end to hepatocytes. However, our studies now show that this is clearly incorrect. We have found that FX acts as a natural defense for Ad virions, shielding the virions from attack by innate natural antibodies and complement. When normal WT mice are depleted of coagulation factors using warfarin, liver transduction by Ad vectors is reduced by several orders of magnitude. In contrast, we found excellent liver transduction in antibody-deficient mice even when coagulation factors were depleted. This finding was further confirmed using two hexon mutant Ads (AdBAP or AdHVR7) that are unable to bind FX. When we restored IgM in antibody-deficient mice, liver transduction by Ad was once again dependent on FX. We also found the same phenomenon in complement-deficient mice that lack proteins of the classical complement pathway (C1q-KO and C4-KO). These data show clearly that FX plays no direct role as a bridge for Ad to transduce hepatocytes. In further work in vitro, we examined interactions between Ad, FX and complement. Using ELISAs, we found that Ad could bind IgM and complement proteins, but not IgG. Importantly, these bindings could be inhibited by FX. In conclusion, our studies for the first time show that the true role of FX is to protect Ad from IgM and complement. This finding is very important for mutating the Ad hexon from binding to FX and detargeting Ad from liver to other tissues for gene therapy.

NICHD
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Doctoral Candidate
Genetics
N-Acetyl Cysteine (NAC) Reverses Hepatic Phenotype of an Antisense Oligonucleotide Mouse Model of Niemann Pick Disease, Type C (NPC): Discovery of a Novel Treatment for NPC
Niemann-Pick Disease, type C (NPC) is an autosomal recessive, lysosomal storage disease characterized by excessive cholesterol and glycosphingolipid storage, and progressive neurological deterioration. Deficiency of either NPC1 or NPC2 leads to failure to efflux unesterified cholesterol and lipids from the late endosome/lysosome (E/L) compartment. As one aspect of the NPC pathological cascade, increased oxidative stress, is supported by a number of in vitro, in vivo, and clinical studies. Accordingly, we hypothesized that N-acetylcysteine (NAC), a prodrug for glutathione, would reduce oxidative stress and provide therapeutic benefit in NPC. To test this hypothesis we utilized a Npc1 antisense oligonucleotide (NPC ASO) mouse model that replicates many aspects of NPC liver disease including liver enlargement, elevated serum transaminase levels, and altered gene expression. This model allows for rapid in vivo testing of candidate therapeutic interventions. To determine if NAC has therapeutic benefit, mice were treated with 1% NAC added to their source of water. In comparison to untreated mice, we observed a significant decrease in the liver to body weight ratio in the NAC treatment group. Transaminase levels were also reduced to control values. Microarray analyses were carried out to investigate mechanistic aspects underlying the reponse to NAC therapy. Of 2249 genes with altered expression in NPC ASO mice, we identified a subset of 92 genes where aberrant expression was corrected by NAC therapy. Taqman gene expression assays validated the results and implied that NAC might play a role in ameliorating several pathological processes involving oxidative stress, proliferation and apoptosis. NAC therapy was also tested in the genetic model in order to study the neurological symptoms. Initial data suggest small increase in life span, and a significant reduction of tremor. This series of experiments demonstrates that NAC treatment addresses a few aspects of the NPC pathological cascade, and thus is a candidate drug for use in the treatment of NPC. Based on evidence from this novel finding, a NAC clinical trial is being conducted in NPC patients at the NIH.

NCI-CCR

Kulkarni, Smita
Postdoctoral Fellow
Genetics
Differential microRNA regulation of HLA-C expression and its association with HIV control
The HLA-C locus is distinct relative to the other classical HLA class I loci in that it has relatively limited polymorphism, lower expression on the cell surface, and more extensive ligand-receptor interactions with killer cell immunoglobulin-like receptors (KIR). A single nucleotide polymorphism (SNP) 35Kb upstream of HLA-C (rs9264942; termed -35) associates with control of HIV, and with levels of HLA-C mRNA transcripts and cell surface expression, but the mechanism underlying its varied expression was unknown. It was proposed that the -35 SNP is not the causal variant for differential HLA-C expression, but rather is marking another polymorphism that directly affects levels of HLA-C. The 3'UTR of the HLA-C gene is predicted to be a target for 26 distinct human microRNAs (miRNAs) of which miR-148a shown to have the greatest likelihood of binding. The binding site for miR-148a contains a single base pair insertion/deletion (ins/del) at position 263 downstream of the HLA-C stop codon. These variants are likely to impose a restriction in miR-148a, as prediction algorithms indicate that the binding of these miRNAs to the alleles marked by 263ins is more stable than to alleles with 263del. Using luciferase assays with vectors containing HLA-C 3'UTRs encoding either 263ins or 263del, we showed direct binding and downregulation of HLA-C 3'UTRs that encode 263ins, whereas HLA-C 3'UTRs encoding 263del escape miRNA mediated down- regulation. We further validated our results by over-expression and inhibition of miR-148a that decreases or elevates cell surface and total protein levels of only the HLA-C alleles that encode 263ins, whereas 263 del encoding HLA-C allele expression remains unaffected. Thus, HLA-C alleles that escape miRNA mediated downregulation are expressed at relatively higher levels. We determined the frequencies of the 263del/ins genotypes in a cohort of 2527 HIV infected European American (EA) individuals. The escape alleles associated strongly with control of HIV viral load. Variation in HLA-C expression adds another layer of diversity to this highly polymorphic HLA-Class I locus that must be considered along with the peptide binding region variants when deciphering the function of these molecules in health and disease. Significant involvement of miRNA in this process provides new approaches for manipulation of the immune system in the treatment of human disease

NHGRI
Ombrello, Michael
Clinical Fellow
Genetics
Genomic Deletions in Phospholipase C-gamma 2 Define a New Syndrome of Cold Urticaria, Antibody Deficiency and Susceptibility to Both Autoimmunity and Infection
Although the coexistence of autoimmunity and susceptibility to infection seems counterintuitive, they are frequent concomitants in immunodeficiency syndromes. Genetic analysis of Mendelian disorders manifesting these seemingly antithetical features may provide novel insights into the molecular basis of this immunologic paradox. We have identified three families with a dominantly-inherited complex of cold-induced urticaria, antibody deficiency, and susceptibility to both autoimmunity and infection. In an effort to better understand the interface between autoimmunity and immunodeficiency, we sought to identify the cause of this phenotype. Cold urticaria was present in every affected member of each family (n=29), while other variable manifestations included antibody deficiency (78%), antinuclear antibodies (56%), sinopulmonary infections (44%), granulomatous skin disease (25%), anti-thyroid autoimmunity (22%), vitiligo (19%), and common variable immunodeficiency (7%). Affected subjects had depressed serum IgM and IgA antibodies, and decreased numbers of circulating class-switched memory B-cells and NK-cells. In vitro assays of B-cell receptor editing, NK-cell degranulation and ligand-induced Ca2+ flux in B- and NK-cells were also impaired. Using SNP genotyping and linkage analysis, we identified a single

7Mb candidate interval on chromosome 16q (LOD=4.2) in one family, which overlapped by 3.5Mb a disease-associated haplotype identified in another family. Given its importance in B- and NK-cells, PLCG2 was selected from our candidate interval for mutational screening. Sanger sequencing of cDNA from purified B-cells revealed PLCG2 transcripts with heterozygous deletions of exon 19 in two families, and exons 20-22 in one family. Long-range PCR and genomic sequencing found three distinct, family-specific deletions in PLCG2 (4.8-8.2kb) that perfectly co-segregated with cold urticaria and were not detected in over 400 healthy control chromosomes. Whole-genome sequencing did not identify other deleterious mutations within the candidate interval. The deletions, which are within an autoinhibitory domain of PLCG2, caused phospholipase hyperactivity, but paradoxically resulted in diminished activation of downstream signaling pathways. We describe a novel immunodysregulatory syndrome in which coding mutations in PLCG2 cause signaling abnormalities in multiple leukocyte subsets and a pleiotropic clinical phenotype encompassing both excessive and impaired immune function.

NHGRI
Rimbault, Maud
Postdoctoral Fellow
Genetics
Fine mapping of two novel loci implicated in canine gigantism
Dog breeds display an extraordinary diversity in phenotypes such as coat color, head shape and body size. No other single mammalian land species approaches this level of diversity. For example, Saint Bernards and Great Danes are up to 100 times heavier than Chihuahuas, and the Greyhound is eight times taller than the Pekinese. Previously our lab found that variation in the IGF1 (insulin-like growth factor 1) gene is a major contributor in determining the body size of small and toy breed dogs. Indeed, a single IGF1 haplotype is shared among all small breeds and nearly absent from large breeds. In order to identify additional loci that affect size, we completed a genome-wide association study (GWAS) using 915 dogs from 80 domestic dog breeds. In contrast to humans where size appears to be governed by numerous small effect loci, our scan revealed only six or seven regions in the dog genome that are associated with major differences in size between dog breeds. Interestingly, only two regions, both on the X chromosome, appear to play a role in increasing size in dogs. The first is strongly associated with "giant" size as displayed by breeds such as the Great Dane and Newfoundland. Breeds are considered giant if they have an average height of at least 26 inches and an average weight of at least 90 pounds, placing them in the top 15% of all domestic dogs. The second locus is most highly associated with a high average weight to height ratio implying a sturdy build. The shared haplotypes identified by the GWAS span 3 Mb for the first locus and 2.2 Mb for the second locus. To identify the gene(s) and the causal mutation(s) that create these phenotypes we are currently fine mapping these two regions through sequencing, SNPs (Single Nucleotide Polymorphism) genotyping and haplotype analysis. The results of this study will contribute to a comprehensive understanding of the mechanisms controlling the full range of body sizes observed in dogs and will also contribute to a better understanding of mammalian growth and morphogenesis.

NCI-DCEG
Tang, Wei
Postdoctoral Fellow
Genetics
Uncommon coding variants within the UGT1A cluster protect from bladder cancer through independent functional mechanisms
Recently, we performed a genome-wide association study (GWAS) and identified a single nucleotide polymorphism (SNP) rs11892031 within the UGT1A gene cluster that belongs to the phase II cellular

detoxification system, as a novel risk factor for bladder cancer. This genetic link is important as it supports exposure to environmental factors such as industrial chemicals and tobacco smoke increase the risk of bladder cancer. The UGT1A locus includes nine protein-coding genes and four pseudogenes and each first exon of these genes is regulated by its own promoter. Due to the complexity of the region and ~90-95% similarity between the substrate-binding exon 1 sequences of UGT1A genes, this region is poorly represented in public databases (HapMap, 1000 Genomes, dbSNP). We started with generating long-range amplicons and sequenced all UGT1A exons in HapMap and bladder cancer patients to ensure specificity of detection and improve the genetic structure of the region. 25 non-synonymous coding variations were identified and genotyped in 2000 bladder cancer samples from the Spanish Bladder Cancer Study. An uncommon coding variant rs17863783 (G>T, Val/Val) within the UGT1A6 was found to be associated stronger than the original GWAS SNP. As bioinformatic tools predicted rs17863783 alleles to differentially bind splicing proteins, we experimentally tested the effect of rs17863783 on splicing and expression of UGT1A6 by creating allelic exontrap minigenes and transiently transfecting into HeLa, 293T, J82 and HepG2 cell lines. The presence of the protective allele T of rs17863783 significantly increased expression of the UGT1A6 compared to with risk G allele. In human normal liver samples expression of UGT1A6 was also increased in carriers of the T allele (p=0.0136). UGT1A6 detoxifies tobacco toxins, environmental and industrial chemicals, which are known risk factors for bladder cancer. Increased expression of UGT1A6 facilitates the removal of the toxins and prevents initiation of carcinogenesis. Our example shows that even uncommon genetic variants can be identified by GWAS and highlights the role of the molecular and genetic mechanisms of gene-environmental interactions and their effects on bladder cancer susceptibility.

NCI-CCR
Thomas, Rasmi
Postdoctoral Fellow
Genetics
Effect of HLA-DP Cell Surface Expression on Hepatitis B Virus Pathogenesis
Background: Clearance of hepatitis B virus (HBV) occurs in 90-95% of infected adults due to effective immune responses, however 5-10% of infected people become chronically infected with HBV leading to cirrhosis and liver cancer. A GWAS study in two Asian populations recently reported a strong genetic predisposition for HBV persistence with HLA-DP haplotypes. We investigated the effect of these DP haplotypes with HBV clearance in the European American (EA) and African American (AA) populations. We also examined the effect of HLA-DP cell surface expression with HBV clearance. Methods: We genotyped the HLA-DP coding gene and 3'UTR regions in 662 HBV-infected individuals of EA and AA ancestry to assess the effect of the reported polymorphism with outcomes of HBV. The allele frequencies, odds ratio (OR) and p values were calculated and computed between the clearance and resistance groups using matched criteria and conditional logistic regression. HLA-DP cell surface expression was characterized using two HLA-DP specific antibodies by flow cytometry in 200 healthy donors. Results: No individual HLA-DPA1 alleles associated strongly with HBV clearance. DPB1*0101 and *0401 associated with HBV persistence and clearance respectively. The variant in the 3'UTR of the DPB gene that strongly associated with HBV clearance in the Asian population GWAS study had only a marginal effect on HBV clearance in the EA and AA populations (CT/TT vs CC; OR=0.41, p=0.01). We identified a novel variant in the DPB 3'UTR region that strongly associated with HBV clearance in these two populations (CT/TT vs CC; OR=0.35, p=0.0001). This variant clearly marked HBV clearance in EA and AA populations as the protective T variant marked *0401 while the susceptible C variant marked *0101 as opposed to the GWAS variant where both these alleles were marked by T. This novel variant also significantly correlated (p=0.0001) with HLA-DP cell surface expression in B cells from fresh PBMCs. Conclusions: A novel variant in the HLA-DPB 3'UTR marks HBV clearance in all populations and is thus

more likely to mark the protective mechanism than the GWAS variant. This novel variant correlates with a significant difference in HLA-DP expression on B cells. We postulate that differential expression of HLA-DP in liver tissue may be more robust and that differences in HLA-DP expression levels may play a role in effective HBV clearance based on individual HLA-DP genotypes.

NHLBI
Ahmad, Shaad
Research Fellow
Genomics
Two Drosophila forkhead proteins control cardiac cell fate and number through polo-dependent cell division
The development of a complex organ requires both the proper differentiation and production of appropriate numbers of each of its constituent cell types, as well as the correct positioning of these cells. We show that jumeau (jumu) and Checkpoint suppressor homologue (CHES-1-like), two genes which encode forkhead (Fkh) family transcription factors, mediate all three of these processes during Drosophila cardiogenesis. jumu was one of 70 novel cardiac mesoderm (CM)-expressed genes we identified through an integrated genetic, genomic, and computational strategy. CHES-1-like, which was examined because of its homology to jumu, is also expressed in the CM. Loss-of-function mutations in either gene exhibit localized changes in cardioblast number, the occasional presence of giant cardioblast nuclei, and misaligned and incorrectly positioned heart cells. Using appropriate markers for different cell types and the products of asymmetric and symmetric cell divisions, together with predictions about the numbers of resulting cell types, we demonstrate that these phenotypes are the result of (i) karyokinesis defects during both types of cell divisions, (ii) asymmetric cell division defects which fail to bring about the derivation of two distinct types of heart cells from their mutual precursor, and (iii) additional symmetric cell divisions that produce yet a third type of cardiac cell. Multiple lines of reasoning led us to suspect that the roles in cell division of both Fkh genes are mediated by acting through a pathway dependent on a kinase encoded by polo, a gene known to be involved in multiple steps in both symmetric and asymmetric cell division: polo was also one of the 70 CM-specific genes identified in our study, and mutations in polo replicate all of the cardiac phenotypes of jumu and CHES-1-like mutants. This hypothesis was confirmed by assays which demonstrated synergistic genetic interactions between jumu and polo, and between CHES-1-like and polo. Moreover, ubiquitous expression of polo was able to partially rescue the jumu and CHES-1-like mutant phenotypes, indicating that the Fkh genes acted upstream of polo in a regulatory pathway. This localized control of a ubiquitous cell cycle regulator by the spatially and temporally restricted expression of two Fkh transcription factors illustrates one possible method for coordinating diverse developmental processes in the construction of a complex organ.

NHGRI
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Visiting Fellow
Genomics
Discovery of new anti-malarial chemotypes, target identification and implication for therapies
Malaria P. falciparum is a lethal infectious disease causing more than one million deaths annually. No effective vaccine is available and parasites have developed resistance to all the current antimalarial drugs. Therefore, identification of new antimalarials is urgently needed. By using a SYBR Green proliferation assay of P. falciparum, we conducted quantitative high throughput screening to test 2,077 compounds from the stereochemically-rich CMLD-BU library against five P. falciparum lines (CP250, Dd2, HB3, 7G8 and GB4) of distinct geographic origin. Among the compounds examined, six showed activities

with IC50 values less than 2 µM across all the parasite lines. Structural analysis demonstrated that these compounds were derived from polycyclic ketal and indoline alkaloid chemotypes. Follow-up studies testing analogs of 192 polycyclic ketal and 199 indoline alkaloid showed improved potency (IC50 less than 1 uM) and significant structure-activity relationship. In addition, four compounds derived from a dihydropyrimidone scaffold exhibited differential chemical phenotypes (DCPs) that showed fivefold or higher IC50 difference between parents of two parasite crosses, Dd2xHB3 and GB4x7G8. By testing these compounds on 67 recombinant progeny generated from the two crosses and utilizing QTL analysis, we were able to map the gene locus controlling the DCPs to a 35-kb segment on chromosome 7 containing pfcrt that encodes a protein responsible for chloroquine (CQ) resistance. To understand whether pfcrt mutaions confer the DCPs, we tested these compounds on parasites with genetically modified pfcrt. The results demonstrated that like CQ, the DCPs identified were also controlled by pfcrt mutations, but in an opposite manner. That means, instead of wildtype, our compounds target PfCRT mutants. CQ was the least expensive, safe and effective drug, but is no longer used in treating malaria due to widespread pfcrt mutations. Our DCP compounds could be good candidates to use in combination with CQ for treating both CQ resistant and sensitive parasites. Remarkably, when tested with a low dosage of CQ (IC15), this combination was 100-fold more potent against CQ-resistant line Dd2 displaying an IC50 value as low as 9 nM. Further, the compounds we identified were not cytotoxic to HepG2, HEK293 and red blood cells. In conclusion, we discovered three novel antimalarial chemotypes which could be potential leads for developing antimalarial drugs or CQ combination therapy.

NHGRI
Kelada, Samir
Postdoctoral Fellow
Genomics
Genomic Characterization of Allergen-Induced Asthma in Mice
Allergic asthma is a common disorder that results from a combination of exposure to allergens and genetic risk factors. Identifying these “gene-environment interactions” is difficult in human studies but can studied through the use of mouse models. We recently developed a model of house dust mite (HDM)-induced asthma that recapitulates key inflammatory endpoints, namely airway eosinophilia and neutrophilia. To identify quantitative trait loci (QTLs) that underlie airway eosinophilia and neutrophilia, we applied our HDM model to developing lines of the Collaborative Cross (CC). The CC is a new and powerful panel of recombinant inbred mouse lines derived from eight-way crosses of classical and wild-derived inbred strains from three sub-species of Mus musculus: domesticus, musculus, and castaneus. We phenotyped 150 unique lines and observed a remarkable range of variation in airway eosinophilia and neutrophilia. After high-density genotyping, we identified QTLs for eosinophilia and neutrophilia on chromosomes 11 and 7, respectively, indicating heritability for these traits. We then measured gene expression using microarrays and mapped >10,000 expression QTLs (“eQTLs”), including both cis- and trans-regulators of gene expression. Interestingly, we identified a “trans-band” on chromosome 2, in which a single locus affects the expression of more than 90 genes in trans. Preliminary analysis suggests that the causal gene in the QTL is a transcription factor, Foxa2. To link the gene expression data with the phenotype data, we identified genes that are associated with each phenotype (using linear regression) and then clustered these genes by the similarity of their expression profiles, yielding “gene regulatory modules” (GRMs). Membership in a GRM implies shared transcriptional control; hence we tested for enrichment of transcription factor (TF) binding sites in each GRM. Novel TF-target gene predictions associated with each trait were generated. For example, a GRM composed key allergen response genes expressed in the airways is enriched for the TF Zeb1. In a completely independent gene expression dataset, we confirmed that the expression of Zeb1 is associated with expression of GRM member genes

(with p-values ranging from 10E-6 – 10E-5). Our results shed light on the role of gene expression in allergic asthma and provide many new and specific hypotheses to further explore in vitro and in vivo.

NHGRI
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Genomics
Diagnosis of Clinically Important Copy Number Variation Using CAPTaIN, A Novel Exome Analysis Software Tool
Copy number variation is an important component of inter-individual genomic variation and can have major biologic and medical consequences. The detection of copy number variation is an evolving field and has recently included the use of whole genome high-throughput sequence read depth as a proxy for copy number. As whole genome high-throughput short-read sequencing is relatively more expensive than whole exome sequencing, and exome sequencing detects many coding-region, potentially highly penetrant (i.e., Mendelian), mutations, exome sequencing is more widely used in biomedical research. However, copy number variation detection from exome sequence data is challenging because of the variability of inter-target capture efficiency, which compounds read depth variability from the sequencing itself. We demonstrate a method to assess copy number variation from exome data that compensates for inter-sample and inter-target read depth variability. This is accomplished using an in silico pooled reference exome, made by calculating average read depths for each target region, which can normalize read depth for test sample comparisons. Deviations from normalized read depths at each region are concatenated into blocks of adjacent read depth ratio aberrations using circular binary segmentation to identify CNVs. We describe the whole exome read pooling process, per sample CNV detection, and validation. These analyses are implemented in a tool called Copy analysis using pooled target intervals for Normalization, CAPTaIN. This tool has been piloted on a set of 210 exomes. Using this approach, we have diagnosed two 1.4 Mb deletions in chr17p11.2, causative of Hereditary Neuropathy with Liability to Pressure Palsies and confirmed a third case previously identified by whole genome sequencing.

NHGRI
Sen, Shurjo
Visiting Fellow
Genomics
Transcriptome Profiling Of Cardiovascular Disease By Massively Parallel Short-Read DNA Sequencing
Background Coronary Artery Disease (CAD) causes more than a fifth of all deaths in the USA. While some genomic markers conferring increased CAD risk have been identified, comparatively little is known about the transcriptomic basis of CAD progression. Knowledge of transcriptomic changes in atherosclerosis, the primary cause of CAD, will improve our understanding of disease progression. Objective To develop methods for studying transcriptomic changes in CAD by combining RNA-Seq and microarrays to identify variation between atherosclerosis cases and controls. Design For eight advanced atherosclerosis subjects and eight age/sex matched controls, we sequenced two transcriptomes from each subject (blood and lymphoblastoid cell line (LCL)) using Illumina next-generation sequencing (NGS). In parallel, we ran Affymetrix expression arrays on RNA from a second LCL growth for each subject, plus an additional set of 16 (eight cases and eight controls). All 32 subjects were sequenced by Agilent whole exome capture and genotyped by Illumina 2.5M SNP arrays. We analyzed sequence and genotype data using custom bioinformatics tools and did statistical tests using BioConductor and JMP. Results LCL RNA-Seq data analysis detected 640 differentially expressed genes between cases and controls (Jensen-Shannon divergence test, p<0.002). GO term enrichment and Ingenuity pathway analysis reveal a

significant enrichment (p<0.003) of genes involved in inflammatory pathways. Affymetrix arrays data from a replicate growth of the LCLs showed a strong positive correlation with the RNA-Seq results (r=0.66). Using a consistency test on the two data sets and applying a 15% FDR cut-off retains 94 genes; 17 of these are independently significant when assayed using both techniques (one-way ANOVA, p<0.05). Interestingly, many of these genes have previous association with CAD based on genomic analyses, but we provide the first evidence that these are also differentially expressed at the RNA level. We detected alternative splicing differences between cases and controls in 53 genes (exon-level ANOVA, p<0.01) as well as allele-specific expression at 60-100 heterozygous SNP positions, including some within CAD-associated genes. Conclusions NGS is a powerful new transcriptome biology tool. We describe the first application of NGS for studying human CAD and report new gene expression and splicing differences that are interesting candidates for follow-up studies in animal models.

NIEHS
Verhein, Kirsten
Postdoctoral Fellow
Genomics
Candidate susceptibility genes in a murine model of RSV-induced bronchiolitis
Respiratory syncytial virus (RSV) is the most common cause of serious lower respiratory illnesses in infants and small children; almost all children have been infected with RSV by age 2. Globally, an estimated 1 million children die annually from severe RSV disease. While genetic association studies have been reported for candidate susceptibility genes for RSV disease, genome wide association studies (GWAS) have not been performed and no biomarkers exist for predicting RSV disease severity. We use a combined genetics and genomics approach to understand the genetic basis of RSV disease susceptibility. We screened 32 inbred mouse strains for response to RSV infection by measuring disease phenotypes, including inflammatory cells and protein concentration in bronchoalveolar lavage, airway mucus, and RSV viral load. Differential disease phenotypes were analyzed using SNPster (Novartis), a GWAS algorithm that identifies statistically significant associations between haplotype and phenotype. Statistically significant and suggestive quantitative trait loci (QTLs) were found for all disease phenotypes. A candidate gene Marco (macrophage receptor with collagenous structure) was identified on chromosome 1, and validated using Marco-/- mice. Furthermore, a non-synonymous coding single nucleotide polymorphism (SNP) was significantly associated with severe disease in a case-control cohort of infants with mild and severe RSV-induced lung disease. Microarray mRNA expression data after RSV infection from lungs of resistant and susceptible mice identified gene transcripts expressed only in susceptible strains, including tumor necrosis factor alpha (Tnf). To identify gene transcripts that predict response to RSV, baseline lung gene transcript expression for 29 inbred mouse strains (Novartis) was correlated to phenotype data from the RSV strain screen using linear regression. These studies identified a battery of gene transcripts that were differentially expressed at baseline and significantly correlated with RSV disease severity including Il1rn (IL-1 receptor antagonist). A test set of gene transcripts developed in the mouse model of RSV disease is being tested in samples from the case-control RSV disease cohort to determine predictability of disease severity in humans. Together, these approaches have identified genetic markers of susceptibility to RSV disease to predict severe responders and potentially provide more effective therapeutic targets.

NHGRI
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Clinical Fellow
Hematology/Oncology, Tumor Immunology, and Therapy
Development of targeted therapy for core binding factor leukemias

Core Binding Factor (CBF) abnormalities are associated with approximately 20% of all acute myeloid leukemia (AML), of which approximately half are further sub-classified as acute myelomonocytic leukemia with eosinophilia, also known as AML subtype M4-Eo. Although CBF leukemias carry a relatively favorable prognosis compared to other leukemias, the toxicity of current therapy is associated with significant morbidity and mortality. The fusion gene CBFb-MYH11 is generated by a chromosome 16 inversion that is present in almost all cases of AML subtype M4-Eo. Previous results from in vitro and in vivo studies have shown that CBFb-MYH11 is a key player in the pathogenesis of inversion 16 leukemia and that the encoded fusion protein, CBFb-SMMHC, binds to RUNX1 with high affinity and dominantly inhibits normal RUNX1 function in hematopoiesis. Thus, small molecule inhibitors of the interaction between CBFb and RUNX1 may have potential therapeutic applications for both inversion 16 leukemia as well as other types of leukemia with aberrant RUNX1 function. We developed a CBFb and RUNX1 bead-based proximity assay in Amplified Luminescence Proximity Homogenous Assay (ALPHA) Screen format and optimized it for high throughput screening. This is the first reported use of this technology for a protein-protein interaction screen. A total of 243,398 compounds at 7 different concentrations each were screened with this assay to identify 137 putative inhibitors by Structure-Activity Relationships, and Curve Class. Confirmatory chemical assays were done to validate compounds of interest. Lead hits have been confirmed in tissue culture cells and in our zebrafish model. One compound statistically significantly reduces leukemic cell burden in our CBFb-MYH11 mouse model and more than doubles life span as compared to saline control. Studies of other promising leads in CBFb-MYH11 and AML-ETO mouse models will be underway soon. One of the compounds has already been in a Phase II clinical trial for an unrelated disease. Because human safety data already exists we will submit a FDA Investigational New Drug application and can reasonably expect to be approved to open a clinical trial for patients with CBF leukemias at NIH relatively quickly.

NCI-CCR
Fares, Joanna
Doctoral Candidate
Hematology/Oncology, Tumor Immunology, and Therapy
Modulation of Myeloid-Derived Dendritic Cell Maturity: Unmasking a Novel Role for the Tumor Suppressor p15Ink4b in Immunity.
p15Ink4b (p15) is a tumor suppressor associated with hematological malignancies. Its inactivation is one of the most prevalent epigenetic alterations reported in up to 80% of all acute myeloid leukemia (AML) patients. p15 is also silenced in 50% of patients diagnosed with myelodysplastic syndromes and its silencing correlates with frequent disease progression into AML. During the leukemogenesis process, escape of pre-leukemic cells from immune clearance represents an important step in the establishment of leukemic disease. Dendritic cells (DCs) are potent antigen-presenting cells that play a critical role in the regulation of immune responses. In immune surveillance, their primary function is to stimulate naïve T cells against pathogens and cancerous cells leading to their effective clearance. However, whether p15 plays a role in DC development has never been addressed. In this study, we found that expression of p15 is strongly induced in a biphasic manner during development and activation of bone marrow-derived DCs (BM-DCs), suggesting an important role for p15 in DC maturation. Also, myeloid-specific deletion of p15 in mice resulted in significantly fewer and less mature myeloid DCs (mDCs) as compared to wild type mice. Consistent with this data, BM cells from knockout mice cultured in vitro generated fewer numbers of BM-DCs that express lower levels of the antigen presenting (MHCII) and the co-stimulatory (CD80, CD86) molecules. Re-expression of p15 resulted in an increase in the expression of both co-stimulatory molecules confirming a role for p15 in the regulation of the maturation process of DCs. Additionally, the incomplete maturation of BM-DCs correlated with a reduced ability to activate T cells in a MHCII-mismatched mixed leukocyte reaction suggesting that loss of p15 affects the function of BM-DCs. Taken

together, our results indicate a novel role for p15 in mDC development, and suggest that frequent inactivation of p15 in myeloid malignancies could lead to an inefficient anti-leukemic immune response during leukemogenesis. Our data also have an important translational significance. AML blasts isolated from patients and differentiated ex-vivo into DCs represent a powerful immunotherapy tool. However, AML-DCs have reportedly a partially impaired maturation process as compared to DCs from healthy donors. We propose that re-expression of p15 in AML-DCs may overcome some of the limitations of a DC-based immunotherapy for AML patients.

NCI-CCR
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Hematology/Oncology, Tumor Immunology, and Therapy
RELB-dependency uniquely distinguishes Hodgkin's Lymphoma from Non-Hodgkin's Lymphomas
Despite considerable evidence supporting the role of the REL members in the immune system and lymphomagenesis, it is not clear whether specific NF-KB dimers control a particular set of target genes that account for the biological functions mediated by these TFs. We performed loss of function experiments delivering specific shRNA to knock down each REL member in different lymphoma cell lines representing stages of germinal center (GC) B cell maturation: Mantle Cell Lymphoma, Diffuse Large B-Cell Lymphoma [DLBCL molecular subtypes: ABC-Like, GCB-Like and PMBL] and Hodgkin Lymphoma (HL) cell lines. All these lymphoma subsets showed a dependency on RELA and c-REL, but only HL cells were sensitive to RELB knock-down. ChIP-seq analysis uncovered an extensive NF-kB network in BJAB (GCB), HBL-1 (ABC), U-H01 (HL) and primary GC B cells. Microarray analysis of these cell lines following knock-down of RELA, RELB, or c-REL was merged with our ChIP-seq data. The overlap of RELB peaks and RELB shRNA downregulated targets suggested that RELB directly controls regulators of the cell cycle, apoptosis, and DNA damage, including CCND3, CDK6, BCL2 and BCL-xL. Additionally, RELB maintains HL cell viability through BCL2 induction: expression of a BCL2 cDNA rescued HL cell lines from the shRNA RELB toxicity. That CCND3, CDK6 and BCL2 are exclusively RELB targets in HL cells is supported by the lack of down-regulation of these genes following knock-down of c-REL. Strikingly, the down-regulation of BCL2 and BCL-xL protein levels in HL cells upon RELB knockdown does not kill these cells by apoptosis as shown by increased cell death by FACS (7-AAD+ cells) but lack of enhancement of Annexin-V+ staining. Furthermore, no cleavage of PARP was detected by western blot. We have morphological and molecular evidence that REL-B inhibits autophagy in HL cells. RELB controls ATG-5 and ATG-7 genes, which play a key role in autophagosomes formation. It has been reported that BCL2 and BCL-xL inhibits this cell death pathway. In summary, we found that HL is the only B-cell malignancy that depends on RELB for viability, and thus differentiates HL from non-Hodgkin lymphomas. These data argue that each REL member has specific and unique functions and targets, as we predicted from the lack of similarities in their transactivation domains. Furthermore, the specificity of the RELB for Hodgkin’s lymphoma makes RELB and the alternative NF-kB pathway an attractive therapeutic target.

NCI-CCR
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Hematology/Oncology, Tumor Immunology, and Therapy
The identification of factors that induce FOXO3 expression and tolerogenic function in tumor associated dendritic cells.
Adoptive T cell transfer immunotherapy is an attractive strategy to treat cancer. However, attempts to utilize this therapy have fallen short due to the loss of T cell function in tumors. To study the complex tumor microenvironment and suppression of immune cells we utilize the TRansgenic Adenocarcinoma of

the Mouse Prostate (TRAMP) model. We have recently demonstrated a novel mechanism that tumor associated DCs (TADC), but not wild type prostate DCs, induce tolerance and suppressive activity in infiltrating T cells. This induction of tolerance is regulated by DC expression of FOXO3, a protein previously identified to be important for cell cycle regulation. Importantly, these results were consistent in human disease. DC isolated from human prostate tumor biopsies were tolerogenic to autologous PBMC, however silencing FOXO3 with siRNAs promoted DC immuno-stimulatory ability. We have now identified two mechanisms that promote FOXO3 expression and tolerogenic functions in DC: 1) factors produced by tumor cells and 2) tumor infiltrating mast cells that produce increased levels of the cytokine IL-13. WT prostate DC cultured in tumor cell supernatant were observed to up-regulate FOXO3 expression and induce tolerance in Ag-specific T cells. Using HPLC, the tumor supernatant was fractionated and used to treat DC prior to testing function. Two of the isolated fractions induced FOXO3 expression and the development of tolerogenic DC. Tolerance promoting sub-fractions were further separated by SPS-PAGE and found to be composed of 12 protein bands of various molecular weights. Individual proteins and combinations of proteins are now being tested for their impact on DC function. Proteins which induce tolerance and FOXO3 expression will then be identified by mass spectrometry. In addition to factors produced directly by the tumor, a second novel mechanism has been identified through the interaction between DC and tumor infiltrating mast cells (MC). MC compose approximately 25% of the infiltrating CD45+ cells in advanced prostate tumors. Upon cross-linking the FcER, purified MC de-granulated and secreted increased levels of IL-13. Further, MC from tumors but not MC isolated from the peritoneum supported the differentiation of FOXO3+ tolerogenic DC via an IL-13 dependent mechanism. Our findings reveal novel mechanisms that promote immune tolerance in favor of anti-tumor immunity and provide promising targets for the enhancement of cancer immunotherapy.

NCI-CCR
Zdanov, Stephanie
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Hematology/Oncology, Tumor Immunology, and Therapy
Tumor cell-extrinsic oncogenic mechanism of mutant Kras in conversion of naïve T cells into Regulatory T cells
Mutations of the Kras oncogene are found in about 25% of human cancers. Although mutated Kras has been shown to be a strong tumor associated antigen, mutated Kras tumors evade immune recognition. In this study, we hypothesized that tumors carrying mutant Kras may promote the inhibition of T-cell immunity by inducing regulatory T cells (Treg) in the tumor microenvironment. Using an in vitro co-culture assay system, we found that mutated Kras tumor cells convert naïve T cells into highly suppressive Treg. Silencing of Kras expression by siRNA in Kras-mutated tumor cells significantly reduced the induction and suppressor activity of Treg. In contrast, tumor cells carrying wild-type Kras generate low numbers of Treg and interference with wild-type Kras expression had no effect on the generation of Treg. We also determined that mutant Kras induces the secretion of Interleukin-10 and Transforming Growth Factor-ß1 through the activation of the MEK-ERK-AP1 signaling pathway and that these cytokines are required for Treg induction. When secretion of these immunosuppressive cytokines was prevented in mutated Kras tumor cells, Treg generation was inhibited. Finally, we observed that colon cancer patients with mutated Kras tumors have an increased number of circulating Treg in comparison with colon cancer patients with wild-type Kras tumors. In conclusion, we identified a novel mechanism for oncogenic Kras in promoting tumorigenesis and revealed that it negatively regulates T-cell immunity by inducing Treg through the secretion of Interleukin-10 and Transforming Growth Factor-ß1. These findings highlight a novel mechanism through which an oncogene can promote oncogenic transformation independent of its transforming activity through facilitating escape from immune recognition.

NIAID-VRC
Johnson, Matthew
Other
HIV and AIDS Research
Determinants or poor immunogenicity of rare-serotype adenoviral vaccine vectors
Several human adenovirus-based vectors of different serotypes are currently under development as potential HIV vaccines. Human adenovirus 5 (Ad5), once considered a leading candidate for a vaccine, stimulates strong immunity, but problems with extensive pre-existing immunity limit its usefulness as a clinical product. Viral vectors derived from rare-serotype adenoviruses, such as Ad28 and Ad35, avoid issues with pre-existing immunity but have proven to be less immunogenic than Ad5-based vectors. We sought to determine which attributes of rare serotype adenoviral vectors inhibit their immunogenicity, or conversely which features of Ad5 increase its immunogenicity. The interaction between adenovirus-derived viral vectors and cells of the human immune system were analyzed using vectors derived from Ad5, Ad28, and Ad35. The level of infectivity of each Ad vector on human peripheral blood mononuclear cells was assessed by polychromatic flow cytometry. The production of cytokines in the media, such as interferon-alpha (IFNalpha), was tested using ELISA. Gene expression array analysis was performed using mRNA isolated from sorted plasmacytoid dendritic cells and myeloid dendritic cells exposed to Ad5, Ad28, or Ad35 vectors. In addition to experiments using primary human cells, the level of IFNalpha induced in mice by the vectors was measured, using both in vivo and in vitro infections. Finally, wild type and interferon alpha-beta receptor knockout transgenic mice were vaccinated with Ad5, Ad28, or Ad35 vectors encoding HIV antigen to determine the impact of IFNalpha production on the generation of antigen-specific T-cell memory. Despite the previously observed decreased immunogenicity of Ad28 and Ad35, our data demonstrate that these serotypes are far more infectious of human primary cells and stimulate greater innate immunity than Ad5. In particular, Ad28 and Ad35 induced IFNalpha production and stimulated interferon-related intracellular pathways. An increase in the in vitro and in vivo production of IFNalpha by Ad28 and Ad35 was found in mice as well. Additionally, we were able to demonstrate that transgenic mice incapable of responding to IFNalpha were able to generate a significantly higher frequency antigen-specific T cell response to Ad28 than wild type mice. Our results indicate vector-induced IFNalpha production may negatively impact adenoviral vector immunogenicity.

NCI-CCR
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HIV and AIDS Research
Characterizing the Role of HIV-1 p6-Alix Binding in HIV-1 Replication.
The central question of how HIV-1 utilizes host cell machinery to enhance virus replication is critically important to our understanding of HIV-1 virus assembly and release. We have molecularly dissected the interaction between HIV-1 Gag and the host cellular ESCRT (Endosomal Sorting Complex Required for Transport)-associated protein, Alix. HIV-1 Gag is responsible for regulating virus assembly. Specifically, the p6 domain of Gag is required for virus budding. Motifs in HIV-1 p6 promote the release of virions from infected cells by interacting directly with host cell factors, such as Alix. Mutational analysis of the HIV-1 p6-Alix binding site demonstrated a functional role of the p6-Alix interaction on HIV-1 replication, Gag polyprotein processing, and particle production. We demonstrated that mutations in the p6-Alix binding site resulted in a profound HIV-1 replication delay in Jurkat T cells. This exciting observation drove us to further ascertain the nature of the replication defect imposed by the p6–Alix binding site mutations. Passaging of the HIV-1 mutants defective for Alix binding led to the emergence of revertant viral isolates that displayed wildtype replication kinetics. Sequencing revealed several putative

compensatory mutations mapping to the HIV-1 Env and Vpu. We confirmed these mutations do indeed compensate for the defects of the p6 mutations on virus replication. We extensively characterized the effects of the mutations on Gag polyprotein processing, Env incorporation, virus release, and single-cycle infectivity in a variety of relevant cell types. These experiments demonstrated that the compensatory mutations in HIV-1 Env and Vpu are coupled to the original HIV-1 p6-Alix binding site mutations. Luciferase reporter assays were employed to quantitate the effects on virus infectivity. Strikingly, we observed an unexpected and significant difference in cell-free versus cell-to-cell virus infectivity in the mutants. Altogether, we hypothesize that defects in cell-to-cell transmission induced by mutations in the p6–Alix binding site are rescued by the compensatory substitutions in Env and Vpu. This study has advanced our mechanistic understanding of the role of Alix in HIV-1 replication. Further characterization of mutations in HIV-1 Vpu and Env will provide novel and important insights into the mechanism(s) by which HIV-1 usurps host ESCRT machinery.

NCI-CCR
Martins, Angelica
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HIV and AIDS Research
Improved HIV-1 Replication Capacity Mediated by Late Domain Duplications in Isolates Carrying Drug Resistance Mutations to Protease Inhibitors.
HIV-1 subverts cellular machinery to allow viral egress from the host cell. The major viral determinant that recruits host cell factors to promote efficient budding from the infected cell is the Pro-Thr-Ala-Pro (PTAP) late domain, located in the p6 region of Gag. The PTAP motif plays a crucial role in virus budding by recruiting components of the cellular ESCRT machinery. Duplications within the PTAP domain occur more frequently in isolates from patients under antiretroviral (ARV) therapy and experiencing treatment failure, than isolates from drug-naïve patients. We analyzed p6 sequences from drug-naive patients and from patients failing ARV therapy. Duplications within PTAP were selected during ARV therapy in HIV-1 subtypes B, F1 and C, the predominant subtypes world-wide. All subtypes accumulated 2 to 2.5 times more duplications in isolates failing ARV. This is the first work describing significant accumulation of PTAP duplications in clinical isolates failing therapy, and we suggest a potential role of the duplications in ARV drug resistance developed by these patients. To address the role of PTAP duplications (PTAPdup) in vitro, we built viruses carrying the duplications with and without drug resistance mutations (DRM) in the protease (PR) gene, and also carrying only the DRM in PR (PRmut). We evaluated the viral release efficiency and viral infectivity in the presence or absence of the PR inhibitor ritonavir (RTV), and morphology of the virus by electron microscopy (EM). Release efficiency of PRmut virus was increased by PTAPdup. Infectivity levels were also increased by PTAPdup in the virus carrying PR DRM in the absence of RTV. Virus carrying only PRmut displayed low infectivity and release efficiency without RTV. In the presence of RTV, the infectivity hierarchy was PTAPdup+PRmut > PRmut > PTAPdup. EM analyses correlated with infectivity results in that virus with PTAPdup+PRmut generated predominantly mature particles, whereas virus with only PRmut had a higher percentage of immature, non-infectious particles. These novel results demonstrate that advantages in viral replication capacity are conferred by PTAP duplications in viruses carrying PR DRM, highlighting the interconnected role of PTAP duplications and PR DRMs in patients' clinical evolution.

NCI-CCR
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HIV and AIDS Research

Xenotropic murine leukemia virus–related virus (XMRV) Probably Originated through Recombination Between Two Endogenous Murine Retroviruses During in vivo Passage of a Human Prostate Cancer Xenograft

XMRV is a gammaretrovirus which is closely related to murine leukemia viruses. It has been recently reported that XMRV is associated with human prostate cancer (PC) and chronic fatigue syndrome (CFS). However, several studies have failed to detect XMRV in patients and have suggested that the positive results can be explained by contamination. A possible origin could be a human PC cell line, 22Rv1, which produces XMRV and was derived from a PC xenograft (CWR22) that was serially passaged in nude mice. To evaluate the genetic variation and evolutionary potential of XMRV, nucleic acid extracts of early and late passages of the CWR22 xenografts were analyzed. DNAs isolated from early and late passage CWR22 xenografts consisted of a mixture of human tumor DNA and nude mouse DNA. It was confirmed that the tumor DNAs were derived from the same person as the 22Rv1 cell line using short-tandem-repeat analysis. Newly developed PCR assays showed that both cell lines and late passage xenografts contained XMRV but the early passage xenografts did not, indicating that XMRV was not present in either the original CWR22 PC or associated nude mouse tissue, but became prevalent in the later passage xenografts. We extensively analyzed xenograft-associated nude mouse DNAs, and isolated two previously undescribed endogenous proviruses, PreXMRV-1 and PreXMRV-2, which contained >3.2-kb stretches of their genomes that were identical (99.92%) to XMRV. Retroviral recombination is a common event during reverse transcription and we found that only few template switching events between PreXMRV-1 and PreXMRV-2 can generate a replication-competent virus that differs from XMRV by only 4 nucleotides over the 8.2-kb genome. To further explore the origin and prevalence of the PreXMRV's, we screened 12 nude mouse strains and found that only the strains that were used to passage the xenograft contained both PreXMRVs, but no XMRV. We conclude that XMRV was not present in the original CWR22 prostate tumor but was generated by recombination between PreXMRV-1 and PreXMRV-2 during in vivo passages of the CWR22 xenograft. The probability of an identical recombinant arising multiple times is vanishingly small, raising the possibility that contamination of human samples with XMRV originating from the 22Rv1 cell line is responsible for its reported association with PC and CFS.

NCI-CCR
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DEVELOPMENT OF ASSAYS FOR IDENTIFICATION OF SMALL MOLECULE INHIBITORS OF VIF-A3G AND VIF-A3F INTERACTIONS

HIV-1/AIDS pandemic continues to remain a global challenge. Despite major strides in the development of antiviral agents, drug-resistant viral variants are rapidly selected in response to all drugs. Hence, novel antiviral agents with diverse mechanisms of action are needed for effective management of HIV-1 infected patients. The recent discovery and characterization of host restriction factors, which provide a potent defense to invading retroviruses, has provided new targets for antiviral drug development. APOBEC3G (A3G) and APOBEC3F (A3F) are potent cytidine deaminases that block HIV-1 replication; HIV-1 expresses the Vif protein, which counteracts A3G and A3F by binding to them and inducing their proteasomal degradation. Mutational studies by our lab and others have identified the minimal regions required for the interaction of Vif with A3G and A3F. The Vif-A3G and Vif-A3F interactions are distinct and specific, and involve different regions of both the A3G/A3F proteins and Vif. These interactions provide two potential targets for development of small molecules which can interfere with the ability of Vif to degrade A3G or A3F. To identify inhibitors that can interfere with Vif-mediated degradation of A3G and A3F, we have developed HeLa cell lines that stably express A3G or A3F tagged with eYFP at the

C-terminal end (A3G-eYFP or A3F-eYFP, respectively) and Vif. In the absence of an inhibitor, Vif induces degradation of A3G-eYFP and A3F-eYFP, and the cells are not fluorescent. In the presence of an inhibitor, it is expected that Vif will be unable to degrade A3G-eYFP and/or A3F-eYFP, resulting in an increase in yellow fluorescence. Quantitative high throughput screening (qHTS) of a small pharmacologically active library by the NIH Chemical Genomics Center (NCGC) identified 4/1395 compounds that interfere with Vif-mediated degradation of A3G (hit rate = 0.29%). Additional primary screen of a small molecule library obtained from Chembridge Inc. identified 18/2960 compounds specific for A3G-Vif and/or A3F-Vif interaction (hit rate = 0.61%). Antiviral testing of these primary hits identified four compounds to have good antiviral activity with minimal cellular toxicity. These studies show that the stable cell lines that we have developed can potentially be used to identify small-molecule inhibitors of the Vif-A3G and Vif-A3F interactions.

NCI-CCR
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HIV and AIDS Research
A Tale of Two HIV-1 Maturation Inhibitors – Bevirimat (BVM) and PF-46396: Insights into Gag Assembly and Virion Maturation
HIV-1 virion maturation is a promising target for antiviral therapy since it is a pivotal step in the production of infectious virions. During maturation, the Gag precursor undergoes viral protease-mediated processing in a kinetically regulated manner, inducing morphological changes in immature particles to form the mature conical core. Thus, the interruption of virion maturation inhibits the production of infectious virions. Bevirimat (BVM), the first-in-class HIV-1 maturation inhibitor, acts by blocking a late step in Gag processing: the cleavage of the capsid-spacer peptide 1 (CA-SP1) processing intermediate to mature CA. Virus particles produced from BVM-treated cells display an aberrant core morphology and a significant loss of infectivity. We identified BVM-resistance mutations in the CA-SP1 boundary region and observed that polymorphisms in SP1 residues 6-8 (QVT) affect BVM susceptibility. Recently, a structurally distinct molecule, PF-46396, was found to elicit antiviral activity by a mechanism of action similar to that of BVM. The lack of structural similarity between the two compounds, however, suggests that they might occupy different binding pockets on assembled Gag, the presumed target of these inhibitors. To investigate this prediction, we performed selections in PF-46396 and identified several resistance mutations clustered in three regions of Gag: the CA-SP1 cleavage site and, more interestingly, two distinct upstream domains in CA. Intriguingly, a group of these CA mutants displayed a striking compound-dependent phenotype; in the absence of PF-46396 these mutants were highly deficient in Gag assembly, with EM analysis revealing patches of Gag accumulating in the plasma membrane of virus-expressing cells. Addition of PF-46396 markedly stimulated particle production and also greatly enhanced the replication capacity of these mutants. We were also able to select second-site revertants for these mutants and observed that the compensatory mutations fell within the same regions to which PF-46396 resistance mapped. Additional analyses defined the consequences to virus maturation of simultaneous treatment with both compounds and also investigated the effect of SP1 QVT motif polymorphisms on susceptibility to PF-46396. These studies provide novel insights into the mechanism of action of HIV-1 maturation inhibitors, and the role of the CA and SP1 in HIV-1 assembly and maturation.

NIAMS
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Immunology - Autoimmune

Interrogation of signaling characteristics associated with C-terminal hypomorphic NEMO mutations reveals a novel regulatory mechanism of innate immune receptor induced NF-kB activation
Activation of the transcription factor NF-kB is a key process in development and host defense and is often upregulated in cancer and inflammatory disease. The NF-kB essential modulator, NEMO, is a key regulator of NF-kB activation. It is aptly termed 'essential' because its genetic deletion results in absent IKK complex activation, impaired NF-kB activation and embryonic lethality. In humans, hypomorphic NEMO mutations (resulting in impaired but not completely absent function) are associated with a heterogeneous Primary Immunodeficiency (PID) termed NEMO Syndrome. The syndrome comprises a broad spectrum of variable developmental and immune function defects, including inflammatory skin and bowel disease which we linked to C-terminal truncations by a comprehensive genotype-phenotype analysis. Receptor activation results in post-translational modifications including phosphorylation, ubiquitination, and deubiquitination. By retroviral reconstitution of NEMO deficient cells and analysis of primary patient samples, we have characterized signaling defects in the inflammatory disease-associated NEMO truncation mutation, E391X. All hypomorphic NEMO mutations reported to date are loss-of-function in that they impair NF-kB activation, consistent with the associated immunodeficiency. However, through detailed biochemical analysis, we have discovered that the NEMO C-terminus is important for negatively regulating NF-kB activation in response to TNF and Toll-like receptor stimulation. By co-immunoprecipitation studies, we have found that IKKbeta T-loop phosphorylation kinetics in response to TNF-alpha, important for kinase complex activation, is normal, but C-terminal serine phosphorylation is increased in E391X cells – indicating the control defect at the level of the IKK complex. By site directed mutagenesis, we further mapped two critical residues in the second proline rich sequence which impair the induced interaction with CYLD, a de-ubiquitinase mutated in a familial cancer syndrome. We are continuing to further characterize the resulting derangement in signaling to gain insight into the precise regulatory function of NEMO. By this forward genetic approach of phenotypic characterization of NEMO Syndrome and related syndromes without a molecular diagnosis coupled with biochemical analysis we seek to gain further insights into fundamental mechanisms by which NF-kB activation is controlled and regulated.

NIEHS
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Immunology - Autoimmune
Cyclooxygenase-2 (COX-2) Negatively Regulates IL-9+/CD4+ T cells (Th9) Differentiation during Allergic Lung Inflammation through down-regulation of IL-17RB
Helper CD4+ T cell subsets, which include Th1, Th2 and Th17 cells, are key components of the adaptive immune response in rodents and humans. Recent data suggests that another distinct helper CD4+ T cell population, Th9 cells, which produce IL-9 and IL-10, exist under certain conditions and that Th2 cells can differentiate into Th9 cells. Our previous studies indicate that COX enzymes play an important role in regulating the Th1-Th2 balance in allergic lung diseases via inhibition of Th2 immune responses. However, the role of COX enzymes in Th9 differentiation is unknown. Herein, using COX-1-/-, COX-2-/- and wild type (WT) mice we investigated whether COX-1 and/or COX-2 were involved in regulating differentiation of Th9 cells during ovalbumin (OVA)-induced allergic lung inflammation. Following OVA sensitization/exposure, the percent of Th9 cells was significantly higher in lung (3.2±0.8 vs. 1.7±0.6), bronchoalveolar lavage fluid (BALF) (3.9±0.5 vs. 2.2±0.7), lymph nodes (27.2±5.1 vs. 14.3±5.1) and blood (20.9±1.9 vs. 13.6±2.3) of COX-2-/- mice, but not COX-1-/- mice, relative to WT controls (all p<0.05). In addition, BALF and serum IL-9 levels were dramatically increased in COX-2-/- mice compared to WT controls. In vitro, Th9 cell differentiation following treatment of naïve CD4+ T cells with TGF-beta and IL-4 was significantly higher in COX-2-/- cells compared to WT cells. Prostaglandin (PG) D2 and PGE2

significantly down-regulated PU.1 expression and inhibited Th9 cell differentiation from naïve CD4+ T cells. PGD2 and PGE2 also inhibited IL-17RB expression, but not IL-17RA, suggesting that PGs directly regulate Th9 cell differentiation through down-regulation of IL-17RB. PGD2 and PGE2 signal through specific EP and DP cell surface receptors. Importantly, Th9 cell differentiation was increased in CD4+ T cells from EP2-/- mice or after siRNA knockdown of DP2 receptor. More importantly, we found human asthmatic patients have significantly increased IL-9+/CD4+ T cells compared with normal controls. PGD2 dramatically inhibited Th9 cell differentiation from human naïve CD4+ T cells. Together, these results indicate that COX-2 inhibits Th9 cell differentiation from naïve CD4+ T cells via PG signaling and regulation of IL-17RB expression in CD4+ T cells. Therefore, targeting COX-2 and/or prostaglandin signaling pathways may represent a novel approach to the treatment of human allergic lung disease.

CC
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Immunology - Autoimmune
Somatic KRAS mutations associated with a human non-malignant syndrome of autoimmunity and abnormal leukocyte homeostasis
Autoimmune lymphoproliferative syndrome (ALPS) is a non-malignant disease characterized by early-onset chronic lymphadenopathy, splenomegaly, multilineage cytopenias, polyclonal hypergammaglobulinemia, expansion of circulating TCRaß+B220+CD4-CD8- T (aß+-DNT) lymphocytes, and an increased risk of B-cell lymphoma. Most ALPS patients have germline or somatic TNFRSF6 (FAS) mutations, and a small minority of patients have germline mutations in the genes encoding FAS ligand and caspase 10. Somatic gain-of-function mutations in members of the RAS subfamily of small guanosine triphosphatases are found in > 30% of all human cancers. We recently reported that an ALPS-like phenotype can be caused by a somatic NRAS mutation (G13D) resulting in the defective lymphocyte apoptosis. Here we demonstrate that somatic mutations in the related KRAS gene can also be associated with a non-malignant syndrome of autoimmunity and breakdown of leukocyte homeostasis. The activating KRAS mutation (G13C) impaired cytokine-withdrawal induced T cell apoptosis through the suppression of the pro-apoptotic protein BIM and facilitated proliferation through p27kip1 downregulation. These defects could be corrected by MEK1 or PI3K inhibition in vitro. We suggest the use of the term RAS-associated autoimmune leukoproliferative disease (RALD) to differentiate this disorder from ALPS. In the hematopoietic system, somatic KRAS and NRAS mutations are commonly observed in aggressive tumors such as multiple myeloma or juvenile myelomonocytic leukemia (JMML). In summary, we identified a new clinical disorder characterized by autoimmunity and benign lymphoproliferation in the presence of somatic mutations in KRAS and NRAS. The therapeutic targeting of signaling pathways downstream of these proteins is a future option for these patients.

NCI-CCR
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Immunology - Autoimmune
Soluble IL7 receptor serves as a depot to prevent excessive IL7 consumption
Background: IL7 signals through a heterodimeric receptor comprised of the a-chain (IL7Ra) and the common ?-chain (IL2RG). The rs6897932 single nucleotide polymorphism in the IL7Ra coding gene modulates susceptibility to multiple sclerosis (MS). The allele associated with increased MS risk also increases an alternatively spliced mRNA isoform lacking exon6. This mRNA isoform encodes a soluble receptor with a unique 26 amino acid stretch at its C-terminus. Thus, although genetic polymorphisms that increase soluble IL7Ra levels increase susceptibility to MS, the physiologic role of soluble IL7Ra

(sIL7Ra) remains poorly understood. Objective: Determine the effect of sIL7Ra on IL7 signaling as a first step toward understanding how polymorphisms in IL7Ra modulate risk for MS. Methods and Results: We produced recombinant isoform encoded sIL7Ra and recombinant extracellular IL7Ra (i.e. shed IL7Ra) and measured binding to IL7 using Plasmon Surface Resonance. Both proteins show intermediate affinity binding, with isoform encoded soluble IL7Ra demonstrating 15-fold increased affinity to IL7 (Kd=6.3nM) compared to extracellular IL7Ra (Kd=98nM). We measured the effect of sIL7Ra on IL7 mediated cell survival using 2E8, an IL7 dependent cell line. At early time points (Day 3) we found that sIL7Ra diminished IL7 mediated survival, however at day 9 and 12 the effect is reversed and cells co- cultured with sIL7Ra show increased survival compared to cells cultured with IL7 alone. Conclusions: Soluble receptor competes with membrane bound for IL7 binding. This leads to a decrease in IL7 signaling at early time points, however at late time points the biologic effects of IL7 are enhanced in the presence of soluble IL7Ra. We postulate that soluble IL7Ra serves as a depot for IL7, resulting in a more persistent IL7 signal over time. Increased levels of soluble IL7Ra may increase the risk of MS by maintaining stronger IL7 signals in vivo.

NCI-CCR
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Other
Immunology - General
Nuclear receptor peroxisome proliferator-activated receptor alpha (PPARa) protects the liver from LPS-induced hepatic apoptosis
Fulminant hepatic failure, acute loss of 80% of hepatocyte function, most commonly results from exposure to viral hepatitis or hepatotoxicants and frequently requires liver transplantation in patients who do not respond to supportive treatment. Except for acetaminophen overdose, there are few proactive treatments available to prevent further liver deterioration. The nuclear receptor peroxisome proliferator-activated receptor alpha (PPARa) is known to regulate lipid homeostasis and the synthetic fibrate PPARa ligand WY-14,643 has recently been identified as anti-inflammatory. Given the significant role PPARa plays in liver homeostasis, in particular mitochondrial function, we hypothesized WY-14,643 treatment would be protective in the lipopolysaccharide (LPS)/D-galactosamine (GalN)-induced acute liver injury model of fulminant hepatitis. Pretreatment of mice with WY-14,643 prevented LPS-induced liver injury as measured by histology score and serum ALT and AST levels. Mitochondrial glutathione depletion and subsequent increase in hydrogen peroxide after LPS treatment was similarly blocked with WY-14,643 treatment. The protective effect of WY-14,643 is dependent on PPARa expression as LPS-treated Ppara-null mice displayed liver injury equivalent to LPS-treated wild-type mice. Unlike LPS, WY-14,643 treatment did not protect against liver apoptosis induced with the anti-Fas/CD95 (clone Jo2) antibody suggesting that WY-14,643 treatment alters specific signaling pathways rather than acting as a general anti-apoptotic/anti-inflammatory signal. C-jun N-terminal kinase (JNK) activation by LPS stimulation was blunted with WY-14,643 co-treatment indicating a reduction in inflammatory and apoptotic signals through tumor necrosis factor receptor 1 (Tnfr1). To confirm the hepatocyte as the site of WY-14,643's protective function, primary hepatocytes and macrophages from Ppara-null mice will be treated in culture with WY-14,643. Additionally, protection from LPS-induced liver injury will be assessed in a hepatocyte-restricted constitutively active PPARa mouse model (VP16-PPARa). The identification of a well-tolerated, commonly prescribed class of drugs, the fibrates, as protective in a model of fulminant hepatitis provides new possibilities for active clinical intervention to prevent further hepatic deterioration in patients.

NIEHS
Burgents, Joseph

Postdoctoral Fellow
Immunology - General
CD103+ and CD11b+ pulmonary dendritic cells display distinct migratory properties during steady-state and following allergic sensitization
Pulmonary dendritic cells (DC) sample inhaled antigens and initiate adaptive immune responses upon migration to lung draining lymph nodes (LN). In the lung, there are two major subsets of CD11c+ DCs, displaying high levels of CD103 (CD103 DCs) and CD11b (CD11b DCs), respectively. The migratory properties of these DC subsets remain unclear, in part because fluorescent reagents intended to label lung resident DCs can passively diffuse from the lung to draining LNs where they are taken up by LN DCs. Also, antibodies to chemokine receptors, such as CCR7, are reported to have limited specificity. To overcome these technical difficulties, we have used an alipathic fluorescent dye (PKH26) which stains pulmonary DCs, but does not diffuse to LNs, and we have generated a CCR7-gfp 'knock-in' reporter mouse strain to track display of this receptor. We report here that although both DC subsets take up Alexa fluor-tagged ovalbumin, many more CD103 DCs migrate to draining LNs than do CD11b DCs. In agreement with these differences, CCR7-gfp was expressed on more CD103 DCs and was expressed at higher levels on these cells than on CD11b DCs. This difference in CCR7-gfp expression was confirmed by analysis of CCR7-specific mRNA and by ex vivo chemotaxis of the two DC subsets in response to the CCR7 ligands CCL19 and CCL21. As expected, instillation of LPS into the lung led to the recruitment of CD11b-expressing, monocyte-derived inflammatory DCs. Surprisingly, however, these DCs did not express CCR7-gfp, and very few PKH26-labeled inflammatory DCs migrated from the lung to draining lymph nodes. Previous studies have shown that lung resident CD11b DCs can be derived either from CCR2-expressing monocytes or from CCR2- pre-DCs. Our current findings suggested the possibility that only pre-DC-derived DCs express CCR7 and are able migrate to draining lymph nodes. In agreement with this notion, CD11b DCs from CCR2-deficient mice had higher levels of CCR7 and migrated more readily to lymph nodes than did their counterparts from WT mice. Thus, CD103 DCs comprise the vast majority of migrating pulmonary DCs during both steady state and inflammatory conditions, and the relatively few CCR7-expressing CD11b DCs in the lung are likely derived from pre-DCs. These findings add to our understanding of pulmonary DC mediated immune responses and suggest that the different functions of the two major DC subsets in the lung can be explained in part by their different migratory abilities.

NIAID
Gerner, Michael
Postdoctoral Fellow
Immunology - General
Distinct Spatial Localization of Resident Dendritic Cell Subsets in Lymph Nodes
Dendritic cells (DCs) are essential for uptake and presentation of antigens (Ags) for T cell activation and can be divided into subsets with varying Ag-presentation capacities. It is not clear whether intrinsic differences or variation in spatial localization and preferential Ag access both contribute to differential Ag-presentation by DC subsets. We thus developed a novel approach of combining multi-parameter (6 or 7 color) confocal microscopy with analytical masking to allow direct in-situ visualization of cellular populations identified by complex phenotypic surface markers. We determined the spatial localization of major resident lymph node (LN) DC subsets and observed preferential positioning of certain populations to distinct LN zones. Some DCs physically associated with lymphatic sinuses (LS) and extended dendrites directly into the LS. While small protein Ags can be directly delivered to resident DCs via LN conduits, particulate Ags are thought to be presented via migratory DCs arriving from peripheral sites. Using 2-photon microscopy, we observed the LS-associated DCs actively sampling the LS for particulate Ags and the formation of T cell clusters in zones proximal to the LS after particulate Ag injection. These results suggest the existence of specialized micro-domains for DC subsets within LN and

suggest that LS-associated DC are important for T cell activation to particulate Ags. This research was supported by the Intramural Research Program of the NIH, NIAID.

NCI-CCR
Hanson, Miranda
Postdoctoral Fellow
Immunology - General
Lactococcus lactis expressing IL-27: A potential therapeutic for inflammatory bowel disease
Inflammatory bowel disease (IBD) affects approximately 1.4 million individuals in the United States. Developing precise targeting of therapeutics to the intestine would greatly advance IBD treatment, thus, we aimed to develop a localized delivery of the immunosuppressive cytokine IL-27 that is actively synthesized in situ by the food-grade bacterium, Lactococcus lactis (L.lactis) to treat chronic IBD. Our in vitro studies confirm that engineered L.lactis express bioactive IL-27. In vivo localization studies showed the presence of lactococci mostly in the luminal contents of the GI tract rather than the mucosal tissue. The therapeutic effect of L.lactis-IL-27 was tested on a T cell transfer model of IBD. Administration of L.lactis-IL-27 to diseased mice rescued all individuals, whereas the control vector group all died. In addition, L.lactis-IL-27 mice had normal colon histology, while the control vector mice had extensive inflammation, crypt abscesses, goblet cell loss, and intraepithelial neoplasia. Using a Disease Activity Index (DAI) that reflects several parameters of IBD, L.lactis-IL-27 mice had no appearance of occult blood in stool, stool consistency was nearly normal, while weight loss was partially relieved. Overall, L.lactis-IL-27 mice had a significantly lower DAI than the vector group. To begin to elucidate the protective mechanism of L.lactis-IL-27, we analyzed IL-10 in distal colon homogenate by ELISA. L.Lactis-IL-27 mice had significantly higher levels of IL-10 than control vector mice. We determined through in vitro and in vivo studies that T cell derived IL-10 is necessary for LL-IL-27's therapeutic effect. In addition, following semi-quantitative reverse-transcribed PCR analysis of inflammatory cytokines in distal colons, we observed significant reductions in TNF-alpha, IL-6, IFN-gamma, IL-23, IL-17A/F, ROR gamma t, and IL-4 levels in L.lactis-IL-27 mice relative to control vector mice. These results suggest that therapeutic application of genetically engineered L.lactis-IL-27 may eventually lead to a more effective and safer management of IBD in humans.

NIAID
Li, Zhanzhuo
Visiting Fellow
Immunology - General
CXCR4 Antagonist AMD3100 Enhances Mixed Chimerism in Mice Receiving Allogeneic Bone Marrow Transplants with Mild Radiation-free Immunosuppression
In the field of bone marrow transplantation (BMT), mixed chimerism refers to a state where both donor and recipient haematopoietic cells are detected in the circulation. Mixed chimerism can be achieved with allogeneic BMT in recipients prepared by cytoreductive conditioning, and may require as few as 1% donor cells for clinical efficacy (for example for donor-specific tolerance in organ transplantation). Cytoreductive conditioning is thought to enhance chimerism by opening bone marrow niches for donor cells, however this approach is often associated with significant toxicity. We hypothesized that mobilizing recipient cells from bone marrow niches would also promote engraftment but be better tolerated than cytoreduction. We tested this approach using AMD3100, a potent antagonist of the chemokine receptor CXCR4 and an approved agent for mobilizing stem cells from bone marrow in patients undergoing BMT in the setting of certain cancers. To determine pharmacokinetics and tissue distribution of the drug, we subcutaneously injected Cu64-labeled AMD3100 w/wo excess unlabeled AMD3100 into WT C57BL/6 (B6) mice. In the blood, radioactivity peaked at 2 hours after injection and

declined quickly thereafter, whereas in thymus, spleen, lymph node and bone marrow drug clearance appeared to be delayed about 10 hours. Accordingly, we injected saline as control or 10 mg/kg AMD3100 at 1 and/or 10 hours before infusing 20 million donor BM cells from Balb/c (Bc) mice into B6 recipients that were conditioned transiently with CD4/CD154 blockade, CD8 depletion and rapamycin, without radiation. Chimerism was determined as the percentage of donor MHC-I-expressing cells in the blood. Uninjected control mice receiving 25 or 50 million donor Bc BM cells attained 0.53 +/- 0.12 and 1.28 +/- 0.3% chimerism, respectively, consistent with published reports. Mice receiving AMD3100 10 hours pre-transplant of 20 million Bc BM cells attained only 0.56 +/- 0.11% chimerism, similar to the mice pre-treated with saline (0.49 +/- 0.09%). In contrast, serial AMD3100 injections at 10 and 1 hour before infusion of 20 million Bc BM cells increased chimerism 3-fold (1.49 +/- 0.17%). Thus, serial injection of AMD3100 10 and 1 hour before allogeneic BMT may enhance mixed chimerism in mice receiving mild conditioning. Additional work will be needed to optimize this regimen and to assess its efficacy in tolerance induction, and in disease settings.

NCI-CCR
Mazor, Ronit
Doctoral Candidate
Immunology - General
Identification and elimination of an immunodominant T cell epitope in a recombinant immunotoxin used to treat cancer
Recombinant immunotoxins (RITs) are genetically engineered proteins, which are being used to treat cancer. They combine the specificity of an antibody with the potent cell killing of a toxin. We have developed RITs that employ a 38kDa fragment of Pseudomonas exotoxin A (PE38) to kill cancer cells. Because PE38 is a foreign protein, only 1 or 2 cycles of immunotoxin treatment can be given before antibodies develop, bind to the PE38 and inactivate the RIT. This prevents additional treatment cycles. Because it is well established that more treatment cycles increase anti-tumor activity, we need to identify and eliminate immunodominant T cell epitopes and produce a new RIT with low immunogenicity that can be given for many treatment cycles. PE38 has 335 amino acids (aa); to span all of the protein we constructed a library of 111 overlapping peptides (15 mers overlapping by 12 aa). PBMCs from 28 naïve donors were collected and genotyped for class II HLA. PBMCs were stimulated with an immunotoxin for 17 days to promote in vitro T cell expansion followed by culture with PE38 peptides for 16 hours. We used an IL2 ELISpot assay to assess responses and mutant peptides with alanine mutations to determine which residues were important for the T cell responses. We analyzed cells from 28 donors from various HLA groups and were very surprised to find only one highly immunodominant region comprised of aa 41-52, with a statistically significant response rate compared to all other regions (P value was less than 0.001). Also the responding cells are all CD4 positive as expected for a class II response. In Silico MHC binding prediction analysis confirmed that this region contains a promiscuous sequence that can bind to over 94 percent of HLA class II proteins. To locate the core aa within the immunodominant peptide, we screened the alanine mutants and found that only 3/10 mutations (L44A, Y45A or R49A), reduced the response by more than 93 percent. We then prepared a new RIT with the R49A mutation and we showed it did not stimulate T cells, yet maintained cytotoxic activity. We conclude that among many individuals with different HLA alleles there is a single dominant T cell epitope in PE38 and that its ability to stimulate the immune response can be abolished by single aa mutations. Therefore it should be possible to use this approach to make new immunotoxins that are more effective in cancer treatment, because more treatment cycles can be given.

NIAID
Arnold, Michelle

Research Fellow
Immunology - Infectious Disease
Determinants of the rotavirus NSP1 protein affecting its role as an interferon antagonist
Rotavirus (RV) is a leading cause of severe childhood diarrhea, annually accounting for approximately 500,000 deaths worldwide. The nonstructural protein NSP1 plays a role in viral spread in the host and thus serves as a potential target of attenuation for improved vaccine design. To suppress the host interferon (IFN) response, many RVs use NSP1 to induce the degradation of IFN regulatory factors IRF3, IRF5 and/or IRF7. Alternatively, some RVs target a component of the complex responsible for activating NF-kB (b-TrCP). The capacity to target different proteins for degradation is consistent with the sequence variation noted among NSP1 proteins, particularly at their C terminus. Despite this variation, all NSP1 proteins share a conserved RING domain near the N terminus formed by a conserved pattern of Cys and His residues, similar to that of some E3 ubiquitin (Ub) ligases. To investigate the role of the RING domain, the variable C-terminal sequence, and conserved Lys residues in the function of NSP1, we engineered mutant NSP1 proteins and assayed their capacity to degrade IRF3, IRF7 and b-TrCP targets. For an NSP1 protein known to target IRFs for degradation (SA11-4F strain), mutation of conserved Cys and His residues of the RING domain interfered with degradation of IRF3 and IRF7. Similarly, for an NSP1 protein known to target b-TrCP for degradation (OSU strain), mutation of conserved Cys residues interfered with degradation of b-TrCP, indicating that the RING domain is key to the activity of NSP1 on any target. Deletion of 17 amino acids from the C terminus of SA11-4F NSP1 also caused the protein to lose the ability to degrade IRF3 and IRF7. Likewise, deletion of the last 13 amino acids of OSU NSP1 resulted in a protein unable to degrade b-TrCP. Thus, the C terminus of NSP1 is required for recognition of all known host targets. Finally, because E3 Ub ligases typically attach Ub to Lys residues of both self and target proteins, we examined the role of conserved Lys residues of NSP1 on the protein's function. Ala substitution of SA11-4F NSP1 Lys residues failed to perturb the ability of the protein to degrade IRF3, suggesting that self-ubiquitination may not be required for NSP1 function. These studies indicate that despite sequence variability, NSP1 proteins from different strains appear to degrade different host targets via a similar mechanism, with the RING domain facilitating the transfer of Ub moieties onto targets recognized by the C terminus of NSP1.

NIAID
Hand, Timothy
Postdoctoral Fellow
Immunology - Infectious Disease
Adaptive immune responses to commensal bacteria during mucosal infection
The mammalian immune system has evolved to maintain tolerance to the large and diverse population of commensal microbes present at mucosal sites. Two main processes are thought to contribute to preventing anti-commensal immune responses: the physical barrier maintained by the gut and active innate and adaptive immune regulation at mucosal sites. This mucosal barrier is critical, as uncontrolled T and B cell responses to mucosal antigens contribute inflammatory bowel disorders. Paradoxically, long-term immune tolerance is maintained during mucosal pathogen infection even though commensal organisms share many immunogenic properties with pathogens or are opportunistic pathogens themselves. Oral infection of mice with Toxoplasma gondii is an illustrative model of a highly inflammatory mucosal infection as it invades,infects and damages the small intestine before escaping to induce systemic infection. We found that T. gondii infection also affects adaptive immune regulation as it induces the deletion of mucosally-associated regulatory T cells (Treg) and thus subverts oral tolerance in response to food antigens. Thus, we hypothesized that during T. gondii infection, tolerance would be lost to the microbiota. In support of this hypothesis, we were measured robust antibody responses against specific commensal bacterial species during infection with T. gondii. As well, TCR transgenic T

cells specific to the flagellum of a common commensal bacteria proliferate and induce transcription factors associated with differentiation, such as T-bet, during T. gondii infection. These systemic adaptive immune responses to commensal bacteria are correlated with robust bacterial translocation to the spleen and liver during T.gondii infection. We found that translocation and commensal-specific immune responses are not specific to T. gondii as they also occur after mucosal infection with the helminth S.mansoni and less pathogenic parasites such as the fungus E.cuniculi. Our data suggest that the 'mucosal firewall' that prevents immune responses to commensal organisms is not absolute and that systemic commensal-specific immune responses are a relatively common occurence. How these commensal-specific T and B cells contribute to the immune response to subsequent challenges at mucosal sites is an important unanswered question. Furthermore, learning how these commensal-specific responses are controlled post-infection may lead to a better understanding of the pathogenesis of IBD.

NIAID
Kindrachuk, Kenneth
Visiting Fellow
Immunology - Infectious Disease
Kinome Analysis Reveals Differential Host Cell Responses to West African or Central African Monkeypox Virus Infection
Background: Monkeypox virus (MPXV) is a member of the genus Orthopoxvirus and causes zoonotic disease with associated case fatality rates of 10-15%. The increasing incidence of MPXV infections and concerns regarding the potential use of MPXV as a bioterrorism agent highlight the importance in determining the molecular mechanisms of disease pathogenesis. Further, infection with viruses from the Western African MPXV clade is associated with less severe infection in humans and non-human primates as compared to the Central African clade. Recent gene expression investigations of host responses to MPXV infection have offered insight into disease pathogenesis. However, many host responses are regulated independently of changes in transcription or translation and are instead regulated through phosphorylation. Thus, global investigations of host kinase activities, i.e. the kinome, through kinome peptide arrays provide a mechanism for investigating disease pathogenesis through the modulation of host cell signal transduction pathways. Here, we sought to compare the cellular response to West African or Central African MPXV infection through kinome analysis. Methods: MPXV Zaire (Central African clade) or MPXV Sierra Leone (Western Africa clade)-infected human THP-1 monocytes were subjected to kinome analysis with peptide arrays representing kinase targets for cell growth and differentiation, stress responses, and innate immunity. Results: Our results demonstrate that MPXV Zaire and Sierra Leone differentially modulate host cell signaling pathways. In comparison to MPXV Sierra Leone, MPXV Zaire repressed pathways related to immune surveillance (FAS signaling pathway), growth and differentiation (FGF signaling pathway, Akt-mediated PI3K signaling events) and apoptosis (PTEN dependent cell-cycle arrest, p53-mediated signaling). Interestingly, MPXV Zaire and MPXV Sierra Leone infection also resulted in the common activation of signaling pathways related to cell proliferation and survival (ErbB signaling pathway, PI3K signaling events). Preliminary confirmatory investigations have demonstrated that MPXV Sierra Leone infection results in increased apoptosis as compared to MPXV. Conclusions: We have demonstrated that infection with MPXV Zaire or Sierra Leone results in differential host cell signaling responses through a novel molecular approach. Importantly, these differences may highlight potential host factor targets for the development of novel antiviral therapeutics.

NCI-CCR
Navarathna, Dhammika

Postdoctoral Fellow
Immunology - Infectious Disease
Candida albicans exploits host eNOS to deregulate host immunity in a mouse model of disseminated candidiasis
Endothelial nitric oxide synthase (eNOS) is constitutively expressed in endothelial cells of mammals, and its highly regulated activity to produce NO is an important regulator of the cardiovascular system. eNOS also show protective influences for the pathophysiology of bacterial infections and parasitic infestations. We studied the role of eNOS in a mouse model of disseminated candidiasis to examine the role of eNOS in a fungal infection. We found that eNOS null mice show a significantly higher survival rate compared with WT mice in a model of disseminated candidiasis caused by a virulent C. albicans strain, SC5314. Kidney and brain are the most important organs in C. albicans infection in both human and mice. Histopathology confirmed that eNOS null mice successfully eliminated invading Candida from the renal cortex with mild inflammatory reactions. An examination of serum cytokines revealed significant elevations of IL-12, IL-17 and the chemokine GM-CSF in eNOS null mice compared with WT at 1, 3 and 5 days post infection .IL-6 levels were significantly elevated in both groups of infected mice, confirming candedemia. However, eNOS null mice showed significantly lower IL-6 levels compared with WT at 5 day post infection. We examined the distribution of neutrophils, monocytes, macrophages, dendritic cells, CD8+ T cell, CD4+ T cells, and natural killer cells infiltrating kidney and brain at 4 days PI by flow cytometry analysis. eNOS null mice had significantly lower neutrophil accumulation in the Candida infected kidneys compared with the WT. To examine changes in the immune regulation, we also studied distribution of the CD4+ T cell Th1, Th2, Th9, Th17 and Treg subsets in spleens of Candida infected eNOS null and WT mice. We found that eNOS null mice have significantly higher Th2 and Th17 cell numbers in the spleen compared with the WT. These results strongly suggest that the lack of eNOS is protective against disseminated candidiasis, associated with reduced kidney invasion and inflammation. A limited neutrophil infiltration may reduce the collateral damage to kidney cortex, while enhanced Th-17 protective immunity contributes to clearance of the invasion. Therefore, an eNOS null background fosters a balanced immune response that is protective against disseminated candidiasis. This represents the first evidence that eNOS has an important role in anti-fungal immunity and we propose that selective eNOS inhibitors would be useful anti-fungal therapeutics.

FDA/CBER
Wang, Yonggang
Research Fellow
Immunology - Infectious Disease
Regulation of A1PI Expression in Monocytes and Macrophages: Potential Role in Anti-Pathogen Responses
Alpha-1 Proteinase Inhibitor (A1PI) is a hepatocyte-derived serine protease inhibitor which covalently binds and blocks neutrophil elastase activity. In patients with constitutively low levels of A1PI, unchecked activity of neutrophil elastase is thought to mediate lung tissue destruction. Some patients with A1PI deficiency display early-onset emphysema and reduced life expectancy. Therapeutic augmentation of serum A1PI has been a standard treatment since 1987. Although A1PI augmentation increases elastase-blocking activity in lung lavage fluid, lung disease still progresses (albeit more slowly) in most patients. For this reason, additional contributions of A1PI to lung homeostasis, beyond elastase inhibition, are being considered. Monocytes and macrophages can express A1PI, but the function is incompletely understood. In recent years, lung macrophages have emerged as key sentinels for infection and injury. To understand potential roles for A1PI in regulating macrophage function, we hypothesized that A1PI modifies inflammatory responses of macrophages in an autocrine and/or paracrine manner. Using ELISA analysis we confirmed that primary monocytes from adults and infants, and monocytic U937

cells, secrete A1PI at high levels (~1 ug/ml). However, using immunoprecipitation analysis, we showed for the first time that A1PI protein is expressed in a non-secreted form by several commonly-used monocytic cell lines (THP-1, MM6, 28SC). Non-secreted A1PI isolated from macrophages demonstrated a molecular mass which is similar to plasma-derived A1PI. Secretion of A1PI by monocytic cell lines was inversely correlated with pro-inflammatory and pro-apoptotic responses to Toll ligands. Proposed intracellular targets of A1PI, such as caspases and hepcidin, have not been previously explored in macrophages and are the focus of current studies. To explore the relevance of macrophage A1PI expression in people, we detected A1PI by immunohistochemistry in lung tissues acquired at autopsy from patients with severe lower respiratory infection. We demonstrated that A1PI was strongly detected and colocalized with CD68 positive cells in patients with cystic fibrosis, and in infants with primary RSV or influenza bronchiolitis. Together our data point to a novel role for A1PI in regulating anti-pathogen responses in monocytes and macrophages. Deficient macrophage responses to respiratory insults may contribute to emphysema in patients with A1PI deficiency.

NINDS
Zinselmeyer, Bernd
Postdoctoral Fellow
Immunology - Infectious Disease
PD-1 Receptor Blockade Promotes Clearance of a Persistent Viral Infection by Overriding Prolonged T cell Engagement
The recent demonstration of improved anti-viral T cell function following PD-1 blockade is a finding of great clinical importance that may ultimately benefit patients with persistent infections and cancer. Presently, it is not known how blockade of this inhibitory pathway improves the function of exhausted T cells. Infection of mice with the immunosuppressive clone 13 strain of LCMV is a well described paradigm for studying mechanisms that cause T cell exhaustion. In fact, the PD-1 pathway was first identified as a mediator of anti-viral T cell exhaustion in this model system. Following clone 13 infection, LCMV-specific CD8 and CD4 T cells progressively lose their antiviral function. In this study we set out to gain novel insights into the mechanisms of T cell exhaustion and recovery during persistent infection by simultaneously imaging splenic CD8 and CD4 T cell responses in real time using two-photon microscopy. We unveiled the mechanism leading to exhaustion by comparing T cell responses to an acute versus a persistent LCMV infection. Motility analyses of T cells operating in these two distinct environments led to the observation that T cell exhaustion during viral persistence is mediated by prolonged target cell engagement in the marginal zone and red pulp; two regions that were heavily infected by clone 13 and expressed an abundance of PD-L1. Importantly, we revealed that during persistent infection PD-1 blockade dramatically enhanced T cell motility within just thirty minutes. This was associated with downstream T cell signaling that did not appear dependent on the TCR, which suggests that the PD-1 pathway can induce motility paralysis in part through a TCR independent mechanism. Enhanced T cell motility was later followed by a massive increase in IFN-g expression (1 hr), a 90% reduction in viral load (16 hrs), and fatal immunopathology (1-3 days). Our novel findings have led us to conclude that the PD-1 pathway reduces T cell functionality (e.g. IFN-g expression) by locking cells into prolonged interactions. Therapeutic blockade of PD-1 breaks these stable interactions and resurrects anti-viral T cell motility and function to the detriment of the host, which ultimately succumbs to severe IFN-g mediated immunopathology. These findings represent a major advance in our understanding of T cell exhaustion and should improve the rationale design of therapies aimed toward improving T cell function in humans.

NCI-CCR
Cuburu, NIcolas
Postdoctoral Fellow

Immunology - Innate and Cell-mediated Host Defenses
Prime/boost genital immunization with human papillomavirus vectors preferentially induces effector-memory CD8+ T cells in the mouse female genital tract by promoting local proliferation of CD8+ T cells upon secondary immunization.
Defining optimal routes of vaccination and developing new vectors for vaccination to achieve strong genital CD8+ T-cell responses may impact the development of vaccines against sexually-transmitted infections. Human Papillomavirus pseudoviruses composed of the viral L1 and L2 proteins can encapsidate virtually any 6-8 kb circular target plasmid. They are attractive gene transfer vehicles, as infectious titers of up to 10(11) can easily be generated in culture. We previously found that intravaginal instillation of Human Papillomavirus vector (HPV) in mice led to transient expression of a reporter gene restricted to epithelial cells, and primed immune responses against a genetically-delivered antigen. Here we studied the dynamics of the CD8+ T-cell responses in the mouse female genital tract after intravaginal HPV prime/boost immunization, using different HPV types in the prime and boost to overcome antibody-mediated neutralization. HPV prime/boost induced several-fold more genital Ag-specific CD8+ T-cells than priming alone or than intramuscular prime/boost with Adenovirus type-5 vector or intranasal immunization with HPV. Most genital CD8+ T-cells were intra- or sub-epithelial whereas no epithelial CD8+ T cells were observed after Ad5 intramuscular immunization. Most of these genital CD8+ T cells displayed effector-memory phenotype, produced TNF-alpha and IFN-gamma simultaneously and were able of cytotoxic activity. Overall genital CD8+ T cell response induced after HPV PsV immunization were durable as readily detected until 6 months post immunization. Using FTY720, a drug that promotes lymph node retention of lymphocytes and in vivo BrdU incorporation assay, we found that the expansion of genital memory CD8+ T-cells upon secondary HPV immunization was due to local proliferation of resident memory CD8+ T-cells and thus may account for the local enhancement of CD8+ T cell responses. These data prompted us to evaluate combinations of intramuscular and intravaginal prime/boost regimen. We found that a systemic prime with Ad5 vector followed by an intravaginal boost with HPV PsV was far superior to any other combination to enhance CD8+ T cell responses in the genital as well as in the systemic compartment. These data underscore HPV vectors as attractive gene-delivery platforms for vaccination to induce long lasting genital CD8+ T-cell memory responses and this approach is currently tested in Herpes simplex virus and vaccinia virus mouse infection models.

NIAID
Grainger, John
Visiting Fellow
Immunology - Innate and Cell-mediated Host Defenses
Control of vitamin A metabolism during infection.
Nutritional status is a critical regulator of immune responsiveness, with malnutrition being the most common cause of immunodeficiency world-wide. Recent work from our laboratory has identified an essential role for vitamin A and its metabolite retinoic acid (RA) in controlling immune responses at large. In particular, in the gastrointestinal tract, we identified the RA / RA-receptor-alpha signaling pathway as a key system not only to promote the generation of regulatory T cells and tolerance at steady-state, but also to drive helper T cell responses when faced with a pathogen, or in the context of oral vaccination. Retinoic acid is generated by a family of enzymes termed retinaldehyde dehydrogenases (RALDH). At steady state, we and others have shown that these enzymes are primarily expressed by a specialized population of CD103+ dendritic cells (DC) residing in the gut, and its associated lymphoid tissue. However, how expression of these enzymes are regulated, and the nature of the cells able to metabolize vitamin A during infection or inflammation has not been addressed. Most importantly, whether populations outside of the gut express RALDH to support developing helper T cell

responses in the periphery remains unknown. Here we use a RALDH-activated fluorescent substrate to identify immune cell populations competent to metabolize vitamin A to RA during oral infection with the highly virulent pathogen Toxoplasma gondii. In sharp contrast to steady-state, in this setting we found that CD103+ DC were no longer the major RALDH expressing population in the gut. This change resulted from the massive recruitment of inflammatory cells, including Ly6C high inflammatory monocytes, that infiltrated the infected gut, and expressed active enzyme. Most importantly, we found that these RALDH+ monocytes infiltrated multiple sites systemically, including tissues like the spleen that do not contain cells capable of processing vitamin A at steady-state. This work provides the first evidence that in the context of inflammation vitamin A metabolism is upregulated systemically by inflammatory cell populations. Based on the primary role of vitamin A in controlling T cell immunity, we believe that this event represents a crucial mechanism to help develop and sustain effector responses at multiple sites during infection or inflammation.

NIAID
Kole, Abhi
Doctoral Candidate
Immunology - Innate and Cell-mediated Host Defenses
TRIF-Dependent Production of Type I Interferons Modulate Inflammatory Responses During T Cell Mediated Colitis
Type I interferons (IFN-1) are a heterogeneous group of cytokines which signal through a shared receptor termed the interferon alpha/beta receptor (IFNAR). IFN-1 are primarily known for their potent anti-viral properties, but their activities in non-infectious contexts are less studied. We found constitutive expression of IFN-1 by MHC II high dendritic cells (DC) and macrophages (MP) from the colons of WT mice, but not mice lacking TRIF, an adapter molecule downstream of the pathogen recognition receptors, toll-like receptor (TLR)-3 and TLR-4. To investigate the role of IFN-1 in intestinal immune homeostasis, we used the T cell transfer model of colitis. Transfer of CD4+CD45RB high naive T cells from WT mice into lymphopenic RAG2-/- mice results in colitis approximately eight weeks post-transfer due to expansion of effector T cells and a relative lack of regulatory T cells. Transfer of IFNAR-/- naive T cells showed similar onset and severity of colitis. In contrast, WT T cells transferred into RAG2-/- x IFNAR-/- mice developed more severe colitis with earlier onset compared to RAG2-/- hosts. IFNAR signaling on hematopoietic cells from RAG2-/- hosts was required to delay colitis development. WT T cells transferred into RAG2-/- x IFNAR-/- mice underwent faster proliferation in the mesenteric lymph nodes and disseminated to the colon earlier than when transferred into RAG2-/- mice. Furthermore, DCs and MPs from the MLNs and colons of IFNAR-/- mice produced less of the anti-inflammatory cytokines interleukin (IL)-10, IL-1R antagonist, and IL-27 but equivalent levels of the pro-inflammatory cytokines IL-1alpha, IL-1beta and IL-23 compared to cells from WT mice upon ex vivo culture. Exogenous IFN-1 did not alter cytokine production by WT DCs and MPs, but antibody blockade of IFNAR decreased production of IL-10, IL-1RA and IL-27. Collectively, these data demonstrate that TLR engagement, most likely by commensal bacterial products, drives constitutive production of IFN-1 by intestinal DCs and MPs in a TRIF-dependent manner. Functionally, this IFN-1 is essential for the production of anti-inflammatory cytokines by intestinal DCs and MPs, and the control of intestinal inflammation in T cell transfer colitis.

NIAID
Kugler, David
Doctoral Candidate
Immunology - Innate and Cell-mediated Host Defenses

Endogenous glucocorticoids play a critical immunomodulatory and host-protective role during Toxoplasma gondii infection

Glucocorticoids (GC) are a class of steroid hormones that play an important role in glucose metabolism. Interestingly, they also exert immunosuppressive effects. As such, GCs are indicated in the treatment of many inflammatory diseases of both Th1 or Th2 etiology. Indeed, much of the suppressive role of GCs is thought to be directed at innate immune cells and their production of proinflammatory cytokines (e.g. IL-1, IL-12). While supplementing GCs may be beneficial for an overly exuberant immune response, it can be detrimental within the context of infection. This has been shown in several experimental models of chronic infection where pathogen reactivation occurs following synthetic GCs administration. Moreover, pathogens themselves are able to induce host GC production, which compromises host immunity. Toxoplasma gondii is an intracellular pathogen that induces a highly polarized Th1 response. It is known that this response, if left uncontrolled, results in immunopathology. Using this experimental model, we observed that T. gondii infection induces high levels of cortisol during the acute phase. We then asked whether the endogenous cortisol response influences the T. gondii-specific adaptive response. To test this, we employed mice that selectively lack glucocorticoid receptor (GR) in T cells. Surprisingly, T. gondii-infected GRfl/fl LCK-cre mice display increased mortality at the peak of acute inflammation compared to wild-type littermates, despite effective parasite control. Indeed, the increased susceptibility of these mice was not due to deficient IFN-gamma production, but rather an over-exuberant Th1 response. In vivo Brefeldin A treatment followed by intracellular cytokine staining revealed a 2-3 fold increase in the percentage of CD4+ IFN-gamma+ positive cells, although there was no significant difference in the frequency of T. gondii-specific T cells as measured by tetramer staining. Importantly, serum IL-12 levels were not different between infected GRfl/fl LCK-cre and control animals. In addition, IL-10, which is known to down-modulate the Th1 response during T. gondii infection, was not impaired but significantly increased in the serum of these mice. Moreover, FACS-purified CD4 T cells from GRfl/fl LCK-cre mice have elevated mRNA levels of IFN-gamma and IL-10. Taken together, our results suggest that endogenous GCs can play an essential role in dampening the CD4 T cell response, by helping maintain a critical threshold for inflammation.

NIAID
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Doctoral Candidate
Immunology - Innate and Cell-mediated Host Defenses

Cutaneous Commensals Control Skin Tissue Immunity

Mammals have co-evolved with an array of bacterial communities that inhabit their barrier surfaces such as the gastrointestinal tract and the skin. While the importance of gut commensals in intestinal immunity is well appreciated, the interaction of organisms resident to other epithelial niches, such as the skin, with the host immune system has not been fully interrogated. To address this, we examined immune cells in the skin tissue of germ free mice that lack all commensal bacteria, fungi, and viruses. Strikingly, we observed a dramatic decrease in the frequency of T cells with the potential to produce inflammatory cytokines IL-17A and IFNg and a concomitant increase in the frequency of Foxp3+ regulatory T cells in skin tissue of germ free mice. We hypothesized that the cutaneous flora locally regulates immune cells in skin tissue. Indeed, perturbation of gut flora resulting in loss of intestinal IL-17A had no impact on cutaneous reservoirs of IL-17A suggesting that skin flora directly regulates cutaneous immunity. Additionally, 16s rDNA phylogenetic analysis of bacteria revealed that murine skin and gut house distinct communities of commensal bacteria. To assess the innate immune factors modulated by indigenous skin flora we evaluated the dendritic cell composition and status of activation. Importantly, we found that the compositions of dendritic cells in the skin of germ free mice were unaltered, however the expression of activation markers CD40, CD80, CD86, and MHCII were

dramatically reduced. Innate inflammatory cytokines that modulate T effector and T regulatory responses such as IL-6, IL-1a, and TNFa were also dramatically reduced in the skin tissue of germ free mice, suggesting that cutaneous commensals control the inflammatory milieu of skin tissue. Further, immunity to the dermal pathogen Leishmania major was abrogated in the absence of commensals and rescued with the association of the cutaneous commensal Staphylococcus epidermidis. Together, these data provide the first insights into the regulation of cutaneous immune cells by resident skin flora and highlight the compartmentalization of immunity in skin tissue.

NIAID
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Immunology - Innate and Cell-mediated Host Defenses
Gastrointestinal eosinophils regulate mucosal CD4+ T cell responses
The gastrointestinal tract (GI) is a dynamic environment in which the immune system must act to maintain tolerance to dietary antigens and commensal flora while remaining poised to respond to pathogens. Tissue resident immune cells of the GI tract are key mediators of this process. Eosinophils comprise a sizeable portion of resident immune cells within the GI tract (>15%). Despite their prominence at this site, the role of eosinophils in the regulation of gastrointestinal immune responses remains unclear. Although eosinophils are typically considered end-stage effector cells of Th2 responses, which are associated with allergy and parasitic infections, their presence during steady state conditions in the GI tract suggests a broader role for these cells in immunoregulation. Thus, we sought to examine the role of intestinal eosinophils in regulating intestinal immunity. To examine the function of this eosinophil population, we made use of a mouse model selectively lacking eosinophils (?dblGATA) as a result of a mutation in the promoter of the GATA1 gene, which is necessary for eosinophil development. Lymphocytes isolated from the intestinal tissue of naïve ?dblGATA mice had a decreased proportion of CD4+ T cells with the ability to produce IL-17A (Th17) and a concomitant increase in CD4+ T cells with the ability to produce IFN-? (Th1). This suggests that gastrointestinal eosinophils infleunce CD4+ T cell polarization and in their absence Th17 responses are impaired. Accordingly, when ?dblGATA mice were infected with the Th17 polarizing bacterial pathogen, C. rodentium, they showed a markedly impaired ability to mount a Th17 response. CD4+ T cells isolated from the colon of infected ?dblGATA mice had decreased capacity to produce canonical Th17 cytokines (IL17A, IL17F and IL22). Importantly, transfer of bone marrow derived eosinophils to ?dblGATA mice largely restored the ability to mount Th17 responses. In an attempt to identify eosinophil derived factors that may affect Th17 polarization, microarray analysis of tissue resident gastrointestinal eosinophils revealed a large upregulation of both IL-1a and IL-1ß as compared to mature eosinophils in the bone marrow. These two cytokines have been shown to be important regulators of Th17 responses in vivo. Thus, eosinophil derived IL-1 may drive Th17 responses in the gut. We are currently investigating mechanisms by which induction of IL-1 in eosinophils may be regulated in the gastrointestinal tract.

NHLBI
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Doctoral Candidate
Immunology - Lymphocyte Development and Activation
Genome-wide analyses of GATA3-mediated gene regulation in distinct T cell types
Transcription factor GATA3 is the master regulator of the T-helper 2 (Th2) subset of CD4+ T cells. GATA3 binds and regulates Interleukins 5 and 13, two of the three signature cytokines of the Th2 lineage. It is essential for CD4+ development from CD4+CD8+ double positive (DP) cells. While Gata3 is expressed in all CD4+ effector lineages, it is not known what GATA3 binds or regulates in non-Th2 subsets. To

elucidate the roles of GATA3 and the mechanisms by which it regulates differentiation, we performed ChIP-Seq profiling of GATA3-binding sites in six T lymphocyte lineages, and RNA sequencing of the same six lineages in wild type/Gata3-knockout pairs. We found that deletion of Gata3 caused widespread, lineage-specific differential expression between wild type and Gata3-knockout cells. Gata3 deletion had an especially strong effect in lineages known to require GATA3 for their development. Of 20,141 transcripts, 1,315 were differentially expressed in early DP, 1,679 in late DP, and 623 in Th2, which are much higher than 52 in iTreg, 24 in Th17, and 200 in Th1. Gata3 knockout-induced differential expression consisted of both lineage-specific activation and repression indicating the complex roles GATA3 plays in developmental transcription networks. We noted that differentially expressed genes in Gata3-knockout cells occurred in immune cell gene interaction networks defined in the KEGG database of gene interaction pathways. In addition to the enrichment of the WGATAR motif in GATA3-binding sites, other motifs were significantly present in different lineages, suggesting a role for co-factors in GATA3 binding. Histone methylation patterns around GATA3-binding sites showed widespread changes in Gata3-knockout DP and Th2 cells. Specifically, we observed a global decrease of H3K4 methylation in 12kb regions around GATA3-binding sites after Gata3 deletion. Genes that were bound by GATA3 and had lower expression after Gata3 knockout showed both a decrease of activating H3K4me and an increase of repressive H3K27me. These observations indicate that GATA3 may have a role in chromatin organization and that this may be the method by which GATA3 regulates expression of its target genes. We have verified that GATA3 is an important regulator of T lymphocyte development and demonstrated its potential mechanisms of action, including co-factors assisting in its binding and its effect on histone modifications, which may control expression.

NIEHS
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Postdoctoral Fellow
Immunology - Lymphocyte Development and Activation
DNA methylation primes the memory B cell epigenome for plasma cell differentiation
Memory is a hallmark of adaptive immunity, wherein lymphocytes mount a superior response to a previously encountered antigen. Upon activation by antigen, naïve B lymphocytes undergo multiple rounds of cell division in the germinal center (GC) before differentiating into plasma cell effectors to secret high affinity antibodies. Alternative to a plasma cell fate, activated B cells also differentiate into memory B cells that can more rapidly acquire effector functions upon subsequent exposure to the same antigen. Memory B cells are quiescent and share a highly similar gene expression profile with their naïve counterparts. It remains incompletely understood how memory B cells can more rapidly turn on a plasma cell differentiation program. It has been speculated that epigenetic alterations in memory cells contribute to their functional distinction from their naïve counterparts. However, the nature and extent of epigenetic alterations in memory compartments remain poorly characterized. In this study, we characterized the DNA methylome of B cell subsets representing various stages of the immune response before and after antigen exposure in vivo. Naïve B cells prior to antigen exposure, activated B cells in GC, post GC-derived memory B cells and plasma cells were purified from inflamed human tonsils for isolation of genomic DNA. Methylated DNA fragments were captured using a methyl-CpG binding protein and analyzed using microarrays covering about 10 percent of the human genome. Comparison of the DNA methylome of B cell subsets indicated that immune activation led to widespread alterations of this epigenetic mark at both promoter-proximal and intergenic regions. These activation-induced DNA methylation changes generated a similar epigenetic signature in downstream memory B cells and plasma cells that is vastly different from naïve B cells. Differential methylated regions between naïve and memory B cells were enriched near genes with immune functions and contain B-cell specific transcription factor binding sites. We propose that the DNA methylation signature of memory B cells

facilitates the recruitment of transcription factors upon antigenic re-challenge, thus allowing them to more rapidly turn on a plasma cell transcriptional program during a secondary immune response.

NIAID
Li, Feng-Yen
Other
Immunology - Lymphocyte Development and Activation
Identification of a novel human primary immunodeficiency with T cell activation defect
The etiologies of primary immunodeficiencies often yield novel insights about the immune system. Although a genetic etiology has been suspected for patients with abnormally low CD4+ T cells in the absence of HIV infection or any known causes of lymphopenia, no genetic mutation has been described to date for any case of primary CD4 lymphopenia. In this study, we characterized a non-consanguineous family with two non-HIV infected boys exhibiting an inverted CD4 to CD8 T cell ratio and a history of recurrent chronic viral infections since birth. Consistent with a decreased thymic output of CD4+ T cells, the percentage of CD31+ cells in the CD4+ naïve population of these patients was decreased. In addition, the activation of T cells was significantly impaired in the patient upon TCR stimulation. Given the mother's T cells show completely skewed X chromosome inactivation, we suspected that the nature of this disease is X-linked. We performed X-chromosome exon-capture targeted single-end Solexa sequencing and found a 10 base pair deletion at an intron-exon junction of a poorly characterized gene described as an ion transporter. We confirmed that this deletion leads to altered splicing, frameshift, early termination of the mRNA, and deficient protein expression in the lymphocytes of the two patients. Moreover, knockdown of this transporter in T cells isolated from healthy donors can recapitulate the observed T cell activation defect while its ectopic expression in the patients' lymphocytes can restore T cell stimulation. Our discovery highlights the significance of this transporter to T cell function.

NCI-CCR
Parish, Stanley
Postdoctoral Fellow
Immunology - Lymphocyte Development and Activation
Unconventional, possibly non-GSL endogenous tumor lipid antigens recognized by CD1d-restricted NKT cells
NKT cells provide a unique niche in the immune system, spanning the bridge between innate and adaptive immunity. Unlike conventional T cells, NKT cells recognize lipid antigens presented by CD1d, and are divided into two types. Type I NKT cells are involved in immunosurveillance, while type II are involved in immunosuppression. Previous work with mouse tumor models showed that both types of NKT cells play a critical role in the regulation of tumor immunity without involvement of exogenous antigens. For example, in cancer models with either CT26 or 15-12RM, NKT cells get activated to suppress tumor immunity, suggesting the existence of endogenous tumor lipid antigens. Although it has been believed that glycosphingolipids (GSLs) are dominant endogenous antigens for NKT cells, little is known about those antigens. To identify endogenous antigens, a two-pronged approach was employed using the same tumor cell lines, CT26 and 15-12. The first arm of this study created a CD1d transfected tumor line containing a thrombin cleavable site (thrombin-CD1d), allowing for the removal of the CD1d/lipid complex without the use of detergents. Both the WT CD1d and thrombin-CD1d transfected 15-12 tumor cells activated both type I and type II NKT cell hybridomas, confirming our hypothesis that an endogenous tumor lipid antigen for NKT cells exists. Furthermore, activation of both types of NKT cell hybridomas by the thrombin-CD1d suggests that the antigen-loading pathway is unaffected by the introduction of a thrombin cleavable site. The second arm involved the use of an in vitro screen for possible lipid antigens in which CD1d dimer/lipid complexes were used to activate both types of NKT

hybridomas. Upon lipid fractionation of CT26 tumor cells, we observe activation in two type I NKT hybridomas within the acidic fraction, which contains predominately GSLs and phospholipids. Loss of activity in the acidic fraction by GSL-enrichment and inability of multiple synthetic forms of GSLs contained in the fraction to activate NKT cell hybridomas unexpectedly suggest that either a non-GSL or unconventional GSL is the predominant lipid antigen that activated the type I NKT cell hybridomas. Ultimately, this work points the field to consider that unconventional, non-GSL, lipids may be the major endogenous tumor antigens responsible for NKT cell activation, and discovery of those lipids could provide the potential targets for cancer immunotherapy involving NKT cells.

NIAMS
Yang, Xiangping
Postdoctoral Fellow
Immunology - Lymphocyte Development and Activation
Competitive Regulation of Interleukin 17 Production in T lymphocytes by the Transcription Factors STAT3 and STAT5
Interleukin (IL)-2 plays a critical role in the maintenance of immune tolerance to self-antigens. Mice and humans that are deficient in the IL-2 receptor develop multi-organ autoimmune disease. Consistent with this, IL-2 is essential for the development of T regulatory cells to maintain the immune tolerance; but in addition, IL-2 inhibits the generation of pro–inflammatory IL-17-producing T lymphocytes (Th17 cells). Despite the association of Th17 cells with a number of autoimmune diseases, the contribution of these cells to the development of inflammatory disease in IL-2 deficient (il-2-/-) mice is not known. To answer this question, il-2-/- mice were bred with mice with T cell specific deletion of the transcription factor STAT3, which is essential for Th17 cell generation. We found that il-2/stat3 doubly deficient mice had significantly less inflammation of the colon and an increased life span compared with il-2-/- mice. Analysis of the colonic T cells showed an increase in IL-17 production in il-2-/- mice, which was abrogated in the absence of T cell STAT3. Next the mechanism of IL-2-mediated Th17 suppression was examined. The T regulatory cell transcription factor, Foxp3 is known to bind and inactivate RORgt, a transcription factor critical for Th17 development. As IL-2 both induces Foxp3 expression and inhibits IL-17, it seemed likely that these two processes were related. By using cells lacking Foxp3, we found that IL-2 inhibits Th17 differentiation independent of Foxp3. However, in the absence of STAT5, we found that IL-2 could not inhibit Th17 differentiation. Conversely, over-expression of a constitutively active STAT5 allele was sufficient to inhibit Th17 differentiation. After stimulation with IL-6 and IL-2, STAT3 and STAT5 bound to multiple common sites across the locus encoding IL-17. The induction of STAT5 binding by IL-2 was associated with less binding of STAT3 at these sites and less active epigenetic marks. Interestingly, 'titration' of the relative activation of STAT3 and STAT5 with different doses of IL-6 and IL-2 modulated the specification of cells to the Th17 cell subset. Overall, the data suggest a novel model of Th17 differentiation and its role in autoimmunity: STAT family members compete with each other for binding to the same gene elements in counter regulation of gene expression, and the balance rather than the absolute magnitude of these signals determines the propensity of cells to become Th17 cells.

NIEHS
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Research Fellow
Informatics/Computational Biology
A meta-analysis reveals novel regulators required for mouse embryonic stem cell self-renewal
Embryonic stem cells (ESCs) can self-renew indefinitely and can differentiate into all derivates (cell types) of the three primary germ layers. Thus, they could one day be exploited for transplantation therapies where a population of cells compromised by a disease/injury could be replaced with new

functional cells. Successful development of such therapies would require a complete understanding of the transcriptional program that maintains the pluripotent genome in a stable state of self-renewal, while allowing rapid induction of alternate transcriptional programs to initiate differentiation. Master transcription factors Oct4, Sox2, cMyc, and Klf4 are necessary for the induction and maintenance of pluripotency. RNAi screens of about 16K genes in mouse ESCs (from 3 studies) collectively have revealed ~400 additional genes with roles in ESC maintenance. While these genome-wide screens were similarly designed and targeted similar sets of genes, there is almost no agreement between the sets of genes reported as required for ESC maintenance. The presence of unique hits in each study suggests that none of these screens have reached saturation and that many additional genes with roles in ESC maintenance remain to be discovered. In an effort to identify the set of all genes required for ESC maintenance, we developed a robust meta-analytic approach to integrate 68 previously published microarray gene expression datasets in mouse ESCs and differentiated cells to generate a list of genes, ranked by a metric quantifying the likelihood of their requirement to maintain ESCs. Top-ranked genes were significantly enriched for known ESC maintenance genes suggesting that genes ranked higher are good candidates for identifying novel ESC regulators. We used RNAi screens to knock-down (KD) 25 previously unscreened genes ranked in the top 500, and discovered 6 novel regulators required for ESC maintenance including Tet1, an enzyme mediating the conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), one uncharacterized transcription factor, two genes involved in rRNA synthesis, one gene known to interact with histones H1 and H3, and one gene involved in histone deacytylation. Our gene expression profiling revealed that at least 2 out of the 6 genes are part of transcriptional circuit controlled by Oct4-Sox2-Nanog. Currently, we are performing high-throughput sequencing to map 5mC and 5hmC levels to understand Tet1's role in regulating DNA demethylation in ESCs.

NLM
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Informatics/Computational Biology
Negative correlation between expression level and evolutionary rates of long intergenic non-coding RNAs
Traditionally, terms “gene”, “coding” etc. have been associated with protein-coding genes, and genomes have been perceived mostly as their repositories. Although this might be largely true for prokaryotes, recent studies on the animal genomes have revealed a vast RNome, i.e. a collection of genes for “non-coding RNAs” (ncRNAs). Strikingly, the number of ncRNAs expessed from mammalian genome exceeds the number of protein-coding genes several-fold. The classification of ncRNAs, whether these loci should be considered genes or not, and their functionality are matters of intensive investigation and debate. Among many distinct classes of ncRNAs, the long non-coding RNAs (lncRNA) are probably the most elusive ones. The definition of lncRNA is based purely on the transcript size: non-coding RNAs longer than 200nt. Their structure is similar to protein-coding genes: many lncRNAs are spliced, 5’capped, and polyadenylated. The functions of the lncRNAs are largely unknown but their evolution appears to be constrained by purifying selection. To gain insights into the mode of evolution of lncRNA, it is natural to compare them with much better characterized protein-coding genes. The rate of evolution of protein-coding genes shows universal negative correlation with expression: genes that are highly expressed in many tissues typically are more conserved during evolution than genes with lower expression level and breadth. This phenomenon is explained by the hypothesis of misfolding-driven protein evolution according to which misfolding is the principal cost incurred by protein expression. We sought to determine if Intergenic lncRNAs (lincRNAs) follow the same evolutionary trend and indeed detected negative correlation between the evolutionary rate and expression level of human and mouse lincRNA genes. Its magnitude is comparable to the correlation of equal-sized sets of protein-coding genes with

similar levels of sequence conservation. Additionally, we found that the expression level of the lincRNAs is positively correlated with the predicted extent of lincRNA folding (base-pairing). However, the contributions of evolutionary rates and folding to the expression level are independent. Thus, the anticorrelation between evolutionary rate and expression level appears to be a general feature of evolution of protein-coding as well as non-protein-coding genes. It might be caused by similar deleterious effects of protein and RNA misfolding or other factors.

NIEHS
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Other
Informatics/Computational Biology
Genome-wide characterization of CTCF's role as an enhancer-blocker
Insulators are DNA elements which when bound by proteins regulate gene expression by blocking interactions between enhancers and promoters, thus limiting the activity of enhancers to certain functional domains. CTCF, the only known insulator-binding protein in vertebrates, has been shown to play diverse regulatory roles including enhancer blocking and genomic imprinting. The region containing H19 and Igf2 genes separated by imprint control region (ICR/insulator) is a well studied locus, where CTCF binds the ICR in a DNA methylation-sensitive manner. Normally, CTCF binds the unmethylated maternal allele ICR, blocking the enhancer downstream of H19 from interacting with Igf2 promoter, thus silencing Igf2 but not H19. On the paternal allele, CTCF is inhibited from binding the methylated ICR, permitting the enhancer to interact with Igf2 promoter, resulting in paternal specific expression of Igf2. Hyper- or hypo-methylation of ICR inhibits/permits CTCF binding resp., resulting in aberrant Igf2 expression associated Beckwith-Wiedemann and Silver-Russell syndromes. In this study, we sought to investigate CTCF's enhancer-blocker role on a genome-wide scale. Previously we had used ChIP-Seq to map CTCF binding sites in HeLa cells, a cancer cell-line. Here, we used ChIP-Seq to map enhancer elements (marked by co-activators and enhancer-specific histone marks) in HeLa cells. We developed an elegant generative grammar-based computational approach to systematically categorize adjacent gene-pairs into 4 groups: (A) those separated by a CTCF site and an enhancer, (B) those separated only by a CTCF site, (C) those separated only by an enhancer, and (D) those that have neither in between. We hypothesized that adjacent genes in group A will be differentially expressed: the enhancer will activate only the gene immediately next to it, with the CTCF-DNA interaction blocking its effect on the other gene. We expected no such effect for gene-pairs in groups B, C, or D. Microarray expression data in HeLa cells revealed that this is indeed the case. To ensure that the observed differences are due to CTCF's direct role is enhancer-blocking, we are currently employing RNAi to knock-down CTCF in an effort to show that the loss of CTCF binding abolishes the expression divergence observed for gene-pairs in group A by essentially converting them into gene-pairs belonging to group C. Our findings would provide a compelling case for CTCF's role as an enhancer-blocker on a global scale.

NLM
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Informatics/Computational Biology
GENOME-WIDE COMPARATIVE ANALYSIS OF PHYLOGENETIC TREES AND SEARCH FOR TRENDS IN THE PHYLOGENETIC FOREST
Comparative genomics has revealed extensive horizontal gene transfer (HGT) among prokaryotes, a development that is often considered to undermine the 'tree of life'(TOL) concept, suggesting that the TOL should be replaced by a 'net of life' as the paradigm of prokaryote evolution. However, the possibility remains that a statistical central trend still exists in the phylogenetic 'forest of life'(FOL).The

FOL encompasses the complete set of phylogenetic trees for all genes from all genomes.This study describes new, cutting-edge methodologies to analyze the FOL and introduces new concepts in prokaryote evolution. We constructed and analyzed all trees of the FOL, which consists of 6,901 phylogenetic trees for prokaryote genes (from 100 species) including 102 nearly universal trees (NUTs).Tree comparison methods have substantially increased in the last few years. However, none of the available methods takes into account the robustness of the branches, a feature is important to minimize the impact of artifacts.Thus, we developed a new method to compare phylogenetic trees, the Boot-Split Distance, that treats tree splits differentially depending on their bootstrap support. A key characteristic of the FOL is the degree of the topological inconsistency among trees.To quantify this trend, we introduced the inconsistency score, as measure of how representative of the entire FOL is the topology of the given tree. A novel method for detecting tree-like and net-like evolutionary trends in the phylogenetic FOL is also introduced.This cutting-edge method involves mapping quartets of species onto trees to identify the tree and net contributions to the distances between species. In conclusion, the analysis of the phylogenetic FOL makes sense given the diversity of gene tree topologies in prokaryotes. Thus, the methodologies introduced in this article may be of wide use to analyze large number of phylogenetic trees. HGT is pervasive among prokaryotes, making the original TOL concept obsolete. However, HGT are distributed randomly and the TOL as central trend is discernible throughout the evolution of prokaryotes. Although diverse routes of net-like evolution collectively dominate the FOL, the pattern of tree-like evolution that reflects the consistent topologies of the NUTs is the most prominent coherent trend. We show contributions of tree-like and net-like evolutionary processes substantially differ across prokaryotic lineages and between functional classes of genes.

NIEHS
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Informatics/Computational Biology
coMOTIF: A Mixture Framework for Identifying Transcription Factor and a Co-regulator Motifs in ChIP-seq Data
ChIP-seq data should be enriched in binding sites for the transcription factor that was immunoprecipitated. Some of the sequences may also contain binding sites for a transcriptional co-regulator. Most existing motif discovery algorithms such as MEME identify one motif at a time. Methods that consider the joint distribution of multiple motifs in the sequences are typically designed to identify clusters of binding sites (a cis-module) that are close to each other, e.g., in a 100- or 200-base pair window. These methods are well-suited for promoter sequences of co-expressed genes on which multiple transcription factor binding sites tend to co-localize. However, they may not be ideal for sequences without well-structured cis-modules such as ChIP-seq data. We developed a finite mixture framework to simultaneously estimate the position weight matrices (PWMs) of both primary and co-regulator motifs and determine which sequences contain both motifs, either single motif, or neither of them. The co-existence of the two motifs in a sequence is modeled by their joint bivariate distribution whereas each single motif is modeled univariately. We use the EM algorithm to iteratively update the parameters of each motif's position weight matrix and the mixing proportions. We compute the posterior probabilities that any given sequence contains both motifs, either motif, or neither, allowing both forward and reverse-compliment orientation. We tested our method on simulated ChIP-seq datasets and showed that it performed better than repeated application of MEME in predicting which sequences contained motifs, and better than a cis-module-based method in estimating the PWMs. We applied our method to a mouse liver Foxa2 ChIP-seq dataset involving ~12,000 400-bp sequences. We identified co-occurrence of Foxa2 with Hnf4a, Cebpa, E-box, Ap1/Maf or Sp1 motifs in ~5-33% of these sequences. All these secondary motifs are either known liver-specific transcription factors or factors

known to play an important role in liver function. This framework is novel in considering the joint distribution of the two motifs within a sequence and estimating the proportion of sequences containing either one or both motifs. Our method is available as a software tool, coMOTIF, and is applicable to large-scale genomic ChIP-seq data for the discovery of a transcription factor and a co-regulator motif.

NLM
Rance, Bastien
Postdoctoral Fellow
Information Science and History of Medicine
Representation of Rare Diseases in Biomedical Terminologies
Motivation: Rare diseases are not always well represented in biomedical terminologies, making it difficult for clinicians to retrieve information from electronic patient records (e.g., for recruitment in clinical trials), for researchers to query bibliographic resources and for patients to navigate health information systems. Materials: We studied the representation of rare diseases in four medical terminologies: the Medical Subject Headings (MeSH), the reference terminology for indexing and searching the biomedical literature; Online Mendelian Inheritance in Man (OMIM), the reference knowledge base about genetic diseases; the Systematized Nomenclature of Medicine-Clinical Terms (SNOMED CT), the world's largest clinical terminology; and the International Classification of Diseases (ICD), used for recording mortality and morbidity statistics worldwide. As a source of rare disease terms, we used the resources created by NIH Office of Rare Diseases Research (ORDR), and the European source Orphanet. ORDR and Orphanet comprise 6857 and 7715 disease concepts, respectively. The Unified Medical Language System (UMLS), a terminology integration system produced by the National Library of Medicine, was used as a pivot between source and target terminologies. Methods: We mapped the rare disease terms from ORDR and Orphanet automatically to the UMLS, used as a pivot to bridge between the rare diseases concepts and the four terminologies under investigation. We evaluated the quality of the results against reference mappings established manually by domain experts. Results: We found a mapping to UMLS for 4881 (71% of 6857) ORDR and 3402 (44% of 7715) Orphanet concepts. Overall, of the unique 7689 rare disease concepts mapped to UMLS, the representation in target terminologies is as follows: MeSH (69%), ICD-10 (11%), OMIM (54%) and SNOMED CT (49%). Based on the 70 reference mappings, the performance of our method is precision 92%, recall 88%, and F-measure 90%. Conclusions: Rare diseases are insufficiently and inconsistently represented in medical terminologies. More than 50% of rare diseases concepts are still not found in such terminologies. Automatic approaches can be used to create a draft mapping and facilitate the work of domain experts. Our results have already been used to improve the mapping of rare disease terms from ORDR.

NCI-CCR
Basseville, Agnes
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Intracellular Trafficking
Histones deacetylase inhibitors mediate rescue of the ABCG2 Q141K variant: potential for a new treatment for gout
Gout is a common chronic form of arthritis that afflicts about 3 million people in the United States. This disease is characterized by high uric acid levels in the blood. 10 % of gout cases are attributable to a polymorphism in the ABCG2 gene. ABCG2 is an ATP-binding cassette transporter, initially known by its involvement in drug efflux, which also mediates renal urate secretion. The Q141K polymorphism impairs the normal expression, localization and function of ABCG2, leading to decreased urate elimination. We have studied the biology underlying altered expression and function of the ABCG2 Q141K variant. We demonstrated that the Q141K variant was fully processed and that the largest fraction was retained in

aggresomes, a cytoplasmic structure where misfolded proteins aggregate. We screened different compounds in order to restore proper trafficking and function of the ABCG2 Q141K variant. The microtubule disruptor colchicine, used in gout treatment, induced a relocalization of the Q141K variant from the aggresome to the cell surface, without affecting protein level. Interestingly, histone deacetylase (HDAC) inhibitors (or HDIs), which are already used for epilepsy treatment and chemotherapy, induced not only a dramatic shift of the Q141K protein from the aggresome to the cell surface, but also restored protein expression to levels similar to the wild-type. This phenomenon was coupled with the increase of ABCG2 specific efflux. We investigated the cellular mechanisms underlying the ability of HDIs to restore ABCG2 Q141K trafficking and function. Phosphorylation, acetylation and dimerization states of ABCG2 Q141K were unmodified after treatment. HDIs induced no change in localization of vimentin, which is part of the aggresome structure. Inhibition of HDAC6, which promotes aggresome formation, did not affect ABCG2 Q141K rescue. Results also showed that HDAC inhibitors rescued only neosynthesized proteins and involved inhibition of retrograde transport. We postulate that colchicine-induced Q141K ABCG2 relocalization involves inhibition of retrograde transport of the variant to the aggresome. In another way, HDI-induced ABCG2 Q141K rescue occurred at multiple levels by modulating the protein transcription, folding and trafficking. These results open doors for a new use of HDAC inhibitors in the treatment of gout and potentially explain the mechanism of action of colchicine.

NIDDK
RAMANATHAN, HARISH
Visiting Fellow
Intracellular Trafficking
The p97 ATPase associates with EEA1 to regulate the size of early endosomes
In endocytosis, internalized vesicles constantly fuse to form early endosomes while maturing into the multivesicular bodies. Endocytic vesicle docking and fusion requires early endosome associated autoantigen 1 (EEA1) that dynamically partitions between endosome-associated and cytosolic forms. Once localized to the membrane, EEA1 functions to tether endocytic vesicles for subsequent fusion catalyzed by NSF and SNARE complex to form early endosomes. Homodimerization of EEA1 is a pre-requisite for its vesicle tethering and oligomerization, which leads to vesicle fusion. Since many internalized cargoes, cellular toxins and pathogens enter the EEA1-containing early endocytic vesicles, regulating EEA1-mediated vesicle tethering and fusion is critical for controlled sorting and delivery of cargoes to different cellular destination or removal from cells. In this study, we took an unbiased biochemical approach and identified p97 as a novel regulator of EEA1 oligomerization. p97 is an abundant, multifunctional cellular AAA-ATPase that plays a well characterized role in ER-associated protein degradation and quality control. Based on its abundance and multifunctionality we predicted that p97 may also control endocytic events. Biochemical fractionation, co-immunoprecipitation (Co-IP), mass spectrometry and deletion mapping experiments demonstrated that p97 is localized to endosomes and physically interacts with EEA1. Genetic depletion of cellular p97 by siRNA leads to enlargement of endosomes in mammalian cells as monitored using confocal and EM experiments. A small molecule p97 inhibitor (EerI) also resulted in similar endocytic size increase. Furthermore, Co-IP experiments using p97-inhibited cell extracts demonstrate increased p97 association with oligomerized EEA1 due to trapping of the enzyme-substrate complex by EerI. Concomitant with the endosome enlargement, trafficking of endocytic cargo in p97-inhibited cells was also drastically delayed. Inducing ER-associated stress response through a p97-independent pathway did not result in endosome enlargement indicating that p97 directly affected the endocytic pathway. Our results support a model in which p97 may regulate the oligomeric state of EEA1 and thereby influence its membrane tethering function, which in turn affects the size of early endosomes. These findings for the first time establish the AAA ATPase p97 as a key regulator of endocytic protein trafficking.

NICHD
Schindler, Christina
Visiting Fellow
Intracellular Trafficking
Understanding BLOC-1 function in cargo sorting towards lysosome-related organelles
In addition to conventional lysosomes, specialized cells such as melanocytes contain lysosome-related organelles (LROs), which fulfill biosynthetic, storage and secretory functions. Biogenesis of lysosome-related organelle complexes (BLOC) are involved in protein trafficking from endosomes to LROs, but not to conventional lysosomes. Mutations in these complexes, or in the endosomal cargo sorting complex Adaptor protein-3 (AP-3), cause a group of disorders referred to as Hermansky-Pudlak Syndrome and characterized by defective LRO biogenesis. The BLOC-1 complex is required to transport critical components of LROs at an early stage of biogenesis, but its molecular function remains elusive. This is largely due to the octameric nature and low intracellular levels of BLOC-1; thus, BLOC-1 has been mainly studied indirectly by depletion of the complex or by analyzing single subunits. In the current study, we expressed and purified octameric BLOC-1 from E. coli and mammalian cells. We found that recombinant BLOC-1 directly and specifically interacts with the core region of AP-3, which is involved in cargo recognition, and the endosomal SNARE proteins Syntaxin13 and SNAP-25, suggesting that BLOC-1 coordinates AP-3-mediated sorting and SNARE-dependent fusion processes. Moreover, BLOC-1 interacts with the cytosolic tails of the melanosome-destined cargo proteins Trp1, Tyrosinase and Pmel17, but not with cargo proteins destined for conventional lysosomes, indicating that BLOC-1 is a co-factor for AP-3-dependent cargo sorting towards LROs. We characterized the overall structure of purified recombinant BLOC-1 by negative staining EM. BLOC-1 is a slightly bent rod, composed of eight globular domains, with length and diameter of 270 and 30 Ångstrom, respectively. The position of individual subunits within the complex is currently being analyzed by placing myc peptide tags on individual subunits and subsequent labeling of these subunits with antibody to the peptide for detection in EM. Future experiments will include mapping binding sites of interacting proteins, which will allow us to generate a model of BLOC-1 function. We will also use recombinant BLOC-1 to identify novel interacting proteins by affinity purification. These studies will contribute to the elucidation of the molecular role of BLOC-1 by establishing a network of interactions and by functionally linking these interactions to well-understood steps in AP-3-dependent cargo sorting and LRO biogenesis.

NIDDK
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Visiting Fellow
Metabolomics/Proteomics
The Potential Role of Serotonin and Myostatin in Metabolism
Myostatin, a secreted protein of the transforming growth factor-beta family negatively regulates skeletal muscle mass. Deletion of the myostatin gene results in increased muscle mass and decreased adipose tissue. In addition, myostatin may regulate metabolism. Myostatin knockout mice fed on a high fat diet gain less weight, have improved glucose tolerance, and are more sensitive to insulin when compared to wild type controls. However, it is not clear whether the reduction in fat and blood glucose levels is a direct or an indirect consequence of altered muscle metabolism. In order to investigate the effects of myostatin on metabolism, we are interested in identifying and characterizing molecules that may be associated with myostatin and metabolism in skeletal muscles. To this end, we carried out a metabolomic screen to identify potential biomolecules that may be altered when myostatin is inhibited in adult mice. Data from serum samples revealed an overall decrease in amino acids when myostatin was inhibited, which is likely due to an increased demand for protein synthesis during muscle

hypertrophy. Furthermore, an overall decrease in fatty acids, accompanied by an overall increase in sugars when myostatin was inhibited, is probably due to increased beta-oxidation of fatty acids and glucose utilization for increased energy production. In addition, other interesting candidates from the metabolomic screen including serotonin were found to be altered when myostatin was inhibited, which paralleled results from a previous unpublished microarray screen performed in our laboratory. The majority of circulating serotonin is produced in the gut (serotonin produced in the brain cannot cross the blood-brain barrier) and has known functions in other tissues including bone formation, insulin secretion by pancreatic beta-cells, and glucose uptake by skeletal muscles. We are therefore examining the effects of myostatin and serotonin on metabolism in skeletal muscle and adipose tissue in both cultured cell lines and in adult mice. Since the aforementioned metabolomic study was performed on serum samples, we are currently investigating whether serotonin levels are altered in skeletal muscle when myostatin is inhibited in adult mice. We hope that this study will not only shed light on normal muscle development and metabolism, but will also suggest potential targets that could be manipulated therapeutically with respect to muscle wasting diseases, diabetes, and obesity.

NIEHS
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Research Fellow
Metabolomics/Proteomics
Proteomic analysis of lipid rafts from ATP Binding Cassette Transporter A1-deficient macrophages reveals novel regulatory events in the innate immune response
Lipid rafts are cholesterol-enriched membrane microdomains that dynamically organize cellular signaling events triggered by extracellular stimuli. Signaling proteins are thought to localize to rafts in part due to raft cholesterol content. The cholesterol efflux transporter ATP Binding Cassette A1 (ABCA1) acts as a primary gatekeeper for eliminating excess cholesterol from the macrophage and its rafts. It is reported that ABCA1 modulates the macrophage's signaling response to bacterial lipopolysaccharide (LPS) through remodeling of raft lipid and protein constituents. However, no proteome-scale analysis of regulation of raft composition by ABCA1 has been reported. Identification of the impact of ABCA1 on the raft proteome of LPS-exposed macrophages carries the promise of identifying novel mechanisms of innate immunity. In order to investigate this question, rafts were isolated using detergent lysis/sucrose density gradient centrifugation from three bone marrow-derived murine macrophage preparations: 1) Abca1+/+ treated with buffer (control); 2) Abca1+/+ treated with LPS (30 min); 3) Abca1-/- treated with buffer; and 4) Abca1-/- treated with LPS. Immunoblotting for raft (flotillin-1) and non-raft (transferrin receptor) targets identified raft fractions displaying no detectable non-raft contamination. Sypro ruby staining of SDS-PAGE-resolved raft samples revealed distinct banding patterns for rafts from the two genotypes, indicating ABCA1-dependent alterations in protein expression. In-gel tryptic digestion of the three raft conditions was carried out, followed by global protein identification using LC-MS/MS and in silico database analysis (Agilent SpectrumMill software). A total 383 proteins were identified across three biological replicates with a minimum of 2 peptides and with a 0.2 % protein FDR and 2% peptide FDR. Several proteins exclusively recruited to rafts have been identified in the LPS-treated and Abca1-deleted samples, including phospholipase C alpha, C5 component of proteosome, and histocompatibility complex. The effect of LPS on expression levels of raft-resident proteins was also examined using spectral count quantitation. A list of five proteins has been identified for further validation by targeted analysis. We conclude that ATP Binding Cassette Transporter A1 regulates the lipid raft proteome of the resting and LPS-stimulated macrophage.

NCI-CCR
Li, Fei

Postdoctoral Fellow
Metabolomics/Proteomics
Metabolomics reveals the mechanism of bile salt export pump mutation related-cholestasis and its novel physiological functions in vivo
Bile Salt Export Pump (Bsep), encoded by ABCB11, is the primary bile salt transporter located at the canalicular domain of hepatocytes. Mutations in the ABCB11 gene result in progressive familial intrahepatic cholestasis type 2 and benign recurrent intrahepatic cholestasis type 2. Bile salts are responsible for the modulation of whole body energy homeostasis through regulation of bile acid transport from the liver to the intestine. Bsep might also have other important roles in mammalian physiology. In an effort to comprehensively understand the physiological functions of Bsep and its mutation related-cholestasis, metabolomics using ultraperformance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry was conducted on urine, serum and liver homogenate from wild-type and Bsep-null mice. Bile acid pool size analysis indicated increased liver bile acids and decreased intestine bile acids in 1.5- to 12-month-old Bsep-null mouse compared with wild-type mice. Serum chemistry showed elevated liver toxicity in Bsep-null mice. Histological analysis revealed the presence of cholestasis in older Bsep-null mice. Liver cholestasis in female Bsep-null mouse was more significant than in male Bsep-null mouse. Metabolomic analysis further showed that 34 endogenous metabolites were significantly modulated in Bsep-null mice. In addition to an increase in bile acids, the metabolomic approach also identified biomarkers for Bsep deficiency previously unassociated with Bsep function: 1) Elevated medium-chain dicarboxylic acids in serum suggested impaired mitochondrial function, consistent with down-regulated genes involved in fatty acid ß-oxidation and up-regulation of oxidative phosphorylation in Bsep-null mouse liver. 2) Increased urinary glycine conjugates and cresol metabolites indicated significant metabolic perturbation in the intestine. 3) An increase in two lysophophatidylcholines (LPC 16:1 and LPC 18:1) in serum and two corticosterone derivatives (HDOPA and DHOPA) in urine demonstrated a defect in phospholipid and corticosterone metabolism. 4) More significant accumulation of bile acids, lysophophatidylcholines, nicotinamide, and HDOPA were observed in female Bsep-null mice. This study suggests that elevated liver bile acids and impaired mitochondrial function are responsible in part for Bsep mutation-induced cholestasis, and that the female may be more sensitive to the Bsep mutations in the clinic compared with males.

NICHD
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Visiting Fellow
Microbiology and Antimicrobials
SidD, a novel deAMPlyase from L. pneumophila
Legionella pneumophila is a Gram-negative bacterium that can cause Legionnaires' disease, a severe pneumonia in humans. Following infection, the pathogen hijacks the intracellular vesicle transport pathway and establishes a camouflaged compartment resembling host cell rough ER for its replication. The bacterium interferes with the early secretory route by several mechanisms, one of which involves exploiting the activity of the small GTPase Rab1, the key regulator of the host cell secretory pathway. The bacterial protein SidM modifies Rab1 through the covalent addition of adenosine monophosphate (AMP), a process known as AMPylation. Overproduction of SidM in tissue culture cells was shown to cause cytotoxicity, an effect that has been attributed to SidM's AMPylation activity. Here we characterized another effector protein SidD from L. pneumophila that catalyzed the removal of AMP from Rab1, a novel activity named de-AMPylation. Using SidD variants with N- or C-terminal truncations in vitro assay we mapped the de-AMPylation activity region to the central part of SidD. COS1 cells producing fluorescently labeled SidD showed partial overlapping localization of SidD to the Golgi,

consistent with the primary localization of its target Rab1. Fragments containing the C-terminus of SidD showed similar localization as full-length SidD. In contrast, no colocalization with specific cellular compartment was detected with SidD fragments lacking the C-terminus, indicating a role of the C-terminus for SidD localization. Cytotoxicity studies revealed that simultaneous production of SidD led to a significant reduction of cytotoxicity in cells producing SidM. Moreover, only the SidD fragment with both the de-AMPylation activity and the C-terminus localization signal is sufficient to rescue the cytotoxicity. Any fragment lacking the C-terminus showed no attenuation of the cytotoxicity due to the mislocalization of the protein. The de-AMPylation reaction catalyzed by SidD was specific for Rab1 because SidD failed to reduce the cytotoxicity caused by AMPylation of Rho GTPases by VopS, an effector protein encoded by Vibrio parahaemolyticus. In this study, we analyzed a new effector protein SidD which exhibited an antagonistic activity of another effector protein SidM towards the same substrate. Our studies added another piece of information on how L. pneumophila effectors could modulate the host cell small GTPase Rab1.

NIAID
Gilk, Stacey
Postdoctoral Fellow
Microbiology and Antimicrobials
Pathogen entry and growth occurs in the absence of host cell cholesterol
Mammalian cell cholesterol has emerged as an important factor in microbial virulence. Pathogens exploit host cell cholesterol as a source of energy and membrane lipid for their respective intracellular compartments. Moreover, several intracellular pathogens, such as Salmonella typhimurium and Chlamydia trachomatis, utilize cholesterol-rich lipid rafts to promote cellular invasion. Studies defining the importance of cholesterol in pathogen-host cell interactions have relied on the cholesterol sequestering agent methyl-beta-cyclodextrin and several cholesterol biosynthesis inhibitors. However, the pleiotropic effects of these compounds complicate experimental interpretation. In the current study, we used cells completely devoid of cholesterol to investigate the importance of the sterol lipid in host cell colonization by three invasive bacteria: Coxiella burnetii, S. typhimurium, and C. trachomatis. We developed a cholesterol-free cell system using DHCR24-/- mouse embryonic fibroblasts (MEFs) that lack the delta24 sterol reductase required for the final enzymatic step in cholesterol biosynthesis. Consequently, these cells accumulate desmosterol as the primary sterol in cellular membranes. Entry of S. typhimurium and C. trachomatis into DHCR24-/- MEFs was unaffected while entry of C. burnetii decreased by approximately 50 percent. Interestingly, once internalized, all three pathogens established their intracellular niche and grew at wild type levels. These data directly contradict dogma that cholesterol is central to pathogen invasion and survival. While not essential for C. burnetii colonization, cholesterol-dependent vesicular trafficking to the C. burnetii-occupied vacuole may occur. We have found that ORP1L, a mammalian cholesterol-binding protein involved in endosomal trafficking, localizes to the C. burnetii vacuole in association with the endoplasmic reticulum (ER). This novel finding suggests the C. burnetii vacuole forms ER contact sites that might facilitate the transfer of lipids. Ongoing experiments are addressing the precise role of ORP1L in C. burnetii vacuole biogenesis and how the pathogen compensates for the lack of cholesterol in DHCR24-/- MEFs.

NHGRI
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Postdoctoral Fellow
Microbiology and Antimicrobials
Genome-wide recombination drives diversification of epidemic strains of Acinetobacter baumannii

Acinetobacter baumannii is an emerging human pathogen and a significant cause of hospital-acquired infections amongst patients in ICU's worldwide. In addition to increasing infection rates, have come an even greater increase in multi-drug resistance among hospital isolates. The escalating drug resistance in A. baumannii has recently culminated in the emergence of strains resistant to all available antibiotics, creating an urgent need to understand how A. baumannii evolves in hospital environments in hopes of limiting its impact. Here, we undertook a genomic study of an outbreak of multi-drug resistant A. baumannii that occurred in 2007 at the National Institutes of Health Clinical Center. Complete genome sequencing using the 454 XLR platform was performed on representatives of each of the three multi-drug resistant strain types present during the outbreak. Comparing their complete genome sequences enabled us to conclude that despite all belonging to the same epidemic lineage, the three strains diverged prior to their arrival to NIH. The simultaneous presence of three divergent strains from this particular lineage supports its increasing prevalence in international hospitals, and suggests an ongoing adaptation to the hospital environment. Further genomic comparisons uncovered that much of the diversification that occurred since the divergence of the three outbreak strains was mediated by the uptake of foreign DNA and subsequent replacement of homologous regions of their chromosomes through homologous recombination. Inspection of specific recombinant regions revealed that several were associated with either the loss or swapping out of genes encoding proteins that are either exposed to the cell surface (e.g. transporters) or that synthesize cell surface molecules (e.g. lipopolysaccharide). Extending our analysis to a more diverse set of international clinical isolates revealed a previously unappreciated ability of A. baumannii to vary surface molecules through horizontal gene transfer, with subsequent intra-species dissemination by homologous recombination. The role of these variant proteins as potential surface antigens suggests that immune evasion may be driving this ongoing diversification. Thus, while previous genomic analyses in A. baumannii have focused on how antibiotic resistance genes are acquired to evade drug-mediated killing, we find genomic evidence that the ability of A. baumannii to evade immune-mediated killing may be of equal importance.

NCI-CCR
Ballachanda, Devaiah
Postdoctoral Fellow
Molecular Biology - Eukaryotic
Brd4 is a novel atypical kinase that phosphorylates the RNA Polymerase II CTD
Productive transcription elongation requires the phosphorylation of Ser2 and Ser5 residues on the carboxy-terminal domain (CTD) of the largest RNA Pol II subunit, RPB1. Phosphorylation of Ser5 is required for mRNA capping while Ser2 phosphorylation is a pre-requisite for the recruitment of mRNA splicing factors to the transcription site. For the past decade, the CDK9/Cyclin T1 complex PTEFb has been considered the principal Ser2 kinase. PTEFb is recruited into the nucleus by Brd4, a bromodomain protein that was originally described as a chromatin adapter. This recruitment is believed to be mainly responsible for the critical role of Brd4 in regulating HIV and HPV transcription. Brd4 also regulates cell growth and suppresses tumor metastasis, with its expression being a prognostic signature for breast cancer survival. Here we show that Brd4 has intrinsic kinase activity which maps to its N-terminal region. Mutational analysis and the distribution pattern of its kinase sub-domains suggest that Brd4 is an atypical kinase, only 40 of which have been currently identified in the eukaryotic kinome. Furthermore, Brd4 directly interacts with the RNA Pol II CTD both in vivo and in vitro in the absence of PTEFb. Using a combination of synthetic CTD peptides, novel kinase inhibitors and specific antibodies, we show that Brd4 specifically phosphorylates the CTD Ser2 residues, while PTEFb requires Ser5 residues to phosphorylate the CTD. Notably, Brd4 phosphorylation of the CTD is only detected by a recently developed, highly specific CTD Ser2P antibody, and not by the H5 antibody conventionally used to detect CTD Ser2 phosphorylation. These findings are validated in vivo by an increase in Ser2 phosphorylation,

observed upon over-expression of a Brd4 mutant which does not recruit PTEFb. Additionally, we show that the Ser2 phosphorylation remains unchanged in bone marrow stem cells and mouse embryonic fibroblasts in which CDK9 is drastically reduced but Brd4 levels are normal. Our results also indicate a strong interplay between the kinase activities of Brd4 and PTEFb that is consistent with their physical interaction reported earlier. Together, these results characterize Brd4 as a hitherto unknown CTD Ser2 atypical eukaryotic kinase and sheds new light on its role in transcription regulation.

NCI-CCR
FUfa, Temesgen
Doctoral Candidate
Molecular Biology - Eukaryotic
Transcriptional Cross-Regulation of the RNA Polymerase II Elongation Factor ELL by MLL-ELL Fusion and HTLV-1 Tax Oncoproteins
RNA polymerase II (Pol II) dependent gene transcription is a complex multistep process that involves pre-initiation complex (PIC) assembly, initiation, elongation and termination. Emerging evidences suggest that specific steps within the pol II transcription cycle are selectively targeted by factors driving pathogenic conditions such as chromosomal translocations and tumor promoting viruses. Here, we describe the eleven-nineteen lysine-rich leukemia protein (ELL) as a regulatory mechanism through which products of leukemogenic chromosomal translocations and viral accessory proteins commandeer and usurp multiple phases of the transcription cycle. ELL is a pol II elongation factor that was initially discovered as a chromosomal translocation partner of the mixed lineage leukemia protein (MLL). Although biochemical and morphological studies in yeast and Drosophila have demonstrated significant insights into the role of ELL in transcription, the mechanisms underlying ELL function in mammalian systems and how its translocation with MLL leads to the development of leukemia remain unclear. We employed combinations of gene depletion, subcellular localization, immunopurification and genome location analyses to demonstrate that ELL bridges dynamic interactions that define two distinct rate-limiting steps in transcription. The first is early targeting and stabilization of the PIC containing Pol II and the histone acetyltransferase p300. The second is stabilization and recruitment of additional transcription elongation factors including the positive transcription elongation factor b (P-TEFb), AFF4 (a frequent fusion partner of MLL in leukemia) and the pol II associated factor 1 (PAF1) to facilitate productive elongation and mRNA maturation. Moreover, we reveal that ELL is a “shared” or common transcriptional target of both MLL-ELL fusion and the human T cell leukemia virus type I (HTLV-1) Tax oncopreteins. Ectopic expression of either MLL-ELL fusion or Tax protein leads to differential targeting of PIC containing ELL/p300 or elongation complex comprised of ELL in association with P-TEFb and AFF4 that culminate with activation of specific genes that promote proliferation. These findings suggest that due to its dynamic role in bridging multiple interactions within the RNA pol II transcription cycle, ELL has evolved as a common cellular target of multiple disease processes and may therefore be a potential candidate for targeted therapy in hematopoietic malignancies.

NIDDK
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Molecular Biology - Eukaryotic
A chaperone holdase maintains polypeptides in soluble state for proteasome degradation
In eukaryotic cells, Endoplasmic Reticulum-Associated Degradation (ERAD) eliminates misfolded proteins to preserve ER homeostasis, which is essential for cell viability. This process requires retrotranslocation of polypeptides in unfolded states by the concerted action of membrane-bound ubiquitin ligases and the translocation-driving ATPase p97. Consequently, polypeptides arriving in the cytosol often bear exposed

hydrophobic motifs or transmembrane domain (TMD), which, if not properly shielded, could cause aggregation to prematurely terminate degradation. How retrotranslocated polypeptides avoid aggregation before reaching the proteasome is unclear. Here we report the identification of a multiprotein complex comprising Bag6, Ubl4A, and Trc35, which associates with ER-bound ubiquitin ligases to chaperone retrotranslocated polypeptides en route to the proteasome. Co-immunoprecipitation experiments show that the Bag6 complex interacts with retrotranslocation complex in the ER membrane, binds retrotranslocated polypeptides, and also associates with the proteasome. Knockdown of each component of the complex attenuates the degradation of several model ERAD substrates. The function of the Bag6 complex in ERAD is revealed by an in vitro biochemical assay using purified recombinant Bag6 and a model unfolded protein Luciferase. We show that Bag6, the central component of the complex, contains an ATP independent chaperone-like activity capable of maintaining the aggregation-prone luciferase in an unfolded yet soluble form. The physiological importance of this holdase activity is underscored by the observation that ERAD substrates accumulate in detergent insoluble aggregates in cells depleted of Bag6, or of Trc35, a cofactor that keeps Bag6 outside the nucleus for engagement in ERAD. Our results establish a model in which Bag6 associates with a retrotranslocation complex to capture dislocated ERAD substrates, which prevents their aggregation in the cytosol prior to degradation. Thus, the Bag6 complex is the first multiprotein chaperone holdase that maintains polypeptide solubility to enhance protein quality control and minimize aggregation in the cell.

NICHD
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Molecular Biology - Eukaryotic
An N-terminal truncated carboxypeptidase E splice isoform induces metastasis by activating nedd9 and other metastasis inducing genes.
Cancer mortality is often from metastatic disease rather than the direct effect of the primary tumor. Identification of metastasis inducing genes may offer valuable mechanistic insight for guiding specific therapeutic strategies. We report here that the carboxypeptidase E gene (CPE) is alternatively spliced in human tumors to yield an N-terminal truncated protein (CPE-DELTA-N) that drives metastasis. CPE-DELTA-N mRNA was elevated in human metastatic colon, breast and HCC cell lines. Suppression of CPE-DELTA-N expression in these cell lines by si-RNA significantly inhibited their growth and invasion. To confirm these observations in vivo, an orthotopic nude mouse model was established. The mice implanted with a tumor derived from HCC cells transfected with si- CPE-DELTA-N RNA in the liver did not show tumor growth or metastasis, compared to scrambled controls. In HCC cytosolic CPE-DELTA-N protein was translocated to the nucleus and upregulated the expression of neural precursor cell expressed, developmentally downregulated gene 9 (Nedd9), through interaction with histone deacetylase (HDAC) 1/2. Inhibition of HDAC activity by the HDAC inhibitors suppressed expression of NEDD9, without effecting CPE-DELTA-N expression. The enhanced invasive phenotype of HCC cells stably transfected with CPE-DELTA-N was suppressed when Nedd9 was silenced by si-RNA. cDNA Microarray studies of HCC cells overexpressing CPE-DELTA-N showed elevated expression of 27 genes associated with metastasis such as Nedd9, claudin 2 (cldn2), carcinoembryonic antigen-related cell adhesion molecule 5 (ceacam5), matrix metallopeptidase 1 (mmp1), plasminogen activator (plat) and inositol 1,4,5-trisphosphate 3-kinase A (itpka), while 30 genes associated with tumor suppressor function, which included insulin-like growth factor binding protein 5 and 3 (igfbp5 and igfbp3) and h19 were down-regulated. These data were further confirmed by qRTPCR. Robust increase in the levels of protranscriptional acetylated histone H3 and H4 in these cells suggest epigenetic regulation of downstream genes by CPE-DELTA-N. Thus CPE-DELTA-N induces tumor metastasis by regulating NEDD9

and other metastasis associated genes expression via interaction with HDAC complex. In clinical studies of 14 patients with thyroid papillary carcinoma, resected tumors having very high copy numbers of CPE-DELTA-N mRNA was correlated with metastasis. Thus CPE-DELTA-N may be a potentially useful biomarker for diagnosing metastasis.

NCI-CCR
TANG, YIZHE
Postdoctoral Fellow
Molecular Biology - Eukaryotic
SRp20 regulates TP53 pre-mRNA splicing and inactivation of SRp20 promotes senescence
Nearly all human genes are alternatively spliced, giving rise to RNA and protein diversity and specificity across tissues, diseases, and development. The tumor suppressor gene TP53 has been shown to have at least 13 splicing variants generating 10 different protein isoforms. p53 is a main regulator of replicative senescence, which is a critical feature of mammalian cells to suppress tumorigenesis working alongside cell death programs. We have shown that senescence-specific p53ß isoform cooperated with full-length p53 to accelerate cellular senescence. However, how p53 pre-mRNA is alternatively spliced in a precisely controlled manner during senescence is unknown. In this study, we tested the role of splicing factor SRp20 in the regulation of TP53 pre-mRNA splicing, and hypothesized that the inactivation of SRp20 is an endogenous regulatory mechanism to promote replicative senescence. Using qRT-PCR and Western blotting, we found that the expression of SRp20 was downregulated in senescent fibroblast MRC-5 and WI-38 cells as well as in isolated senescent T lymphocytes, demonstrating a similar expression pattern of SRp20 in vitro and in vivo. Depletion of SRp20 by siRNA in young fibroblasts dramatically elevated p53ß level and induced cell growth arrest and positive SA-ß-galactosidase staining, which are two classical markers of cellular senescence. To demonstrate that SRp20 regulate p53 pre-mRNA, we performed reverse RNA pull-down assay with biotin-labeled RNA oligos mimicking p53ß exon sequence. The results showed that SRp20 was physically bound to the p53ß unique exon, and the binding regions match the consensus SRp20 binding sites. Mutations of the binding sites abolished such binding of SRp20 on p53ß exon. Since SRp20 could be transcriptionally activated by E2F, based on our findings, we argue that E2F inactivation in the early stage of senescence causes the downregulation of SRp20. Consequently TP53 pre-mRNA is spliced toward p53ß isoform and promotes senescence. Finally, SRp20 was found to be dephosphorylated in colon adenoma tissues (a senescence model system) in contrast to the phosphorylated form in non-tumor tissues, and dephosphorylated SRp20 failed to bind p53ß exon at the consensus regions. Whether dephosphorylation is also part of SRp20 inactivation mechanism is still under investigation. Together, these observations may reveal a new mechanism through which cellular senescence harnesses an RNA splicing program to promote its own progression.

NHGRI
Vilboux, Thierry
Visiting Fellow
Molecular Biology - Eukaryotic
NBEAL2 is mutated in Gray Platelet Syndrome and is required for biogenesis of platelet alpha-granules, and regulates extra-cellular matrix protein expression.
Gray Platelet Syndrome (GPS) is a rare autosomal recessive disorder characterized by bleeding tendency, thrombocytopenia, myelofibrosis and large platelets that lack a-granules. The causative gene has been sought for decades. We mapped the locus for GPS to a 9.4Mb interval on 3p21.1-22.1 that includes 197 protein-coding genes. We sequenced these genes using a combination of next generation and Sanger sequencing in 15 independent GPS families. We identified 15 different mutations in NBEAL2 (neurobeachin-like 2); 5 missense, 4 frameshift, 3 nonsense and 3 splice site mutations. The protein

encoded by NBEAL2 has no known function, but contains 3 domains (BEACH, ARM and WD40) that are also found in LYST, a lysosomal trafficking regulator protein defective in Chédiak-Higashi syndrome. RNA analysis showed that at least 7 NBEAL2 mRNA transcripts are expressed in hematopoietic cells, including megakaryocytes and platelets. Mass spectrometry of discontinuous sucrose gradient platelet fractions identified NBEAL2 localization to the platelet dense tubular system (endoplasmic reticulum). Quantitative RT-PCR, immunoblotting and confocal immunofluorescence microscopy showed that extracellular matrix proteins, including fibronectin and collagen 6, were up-regulated at the RNA and protein levels in all available patients' fibroblasts. We postulate that NBEAL2 may play an important role in controlling the expression of extra-cellular matrix proteins. The aberrant expression and excretion of these proteins could explain the myelofibrosis seen in GPS patients, and can be explored as a therapeutic target.

NCI-CCR
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Molecular Biology - Prokaryotic
RssB phosphorylation, dimerization and interaction with anti-adaptor proteins regulate RpoS proteolysis in E. coli
In response to various environmental stresses, bacteria change their genes expression by activating alternative sigma factors that bind to RNA polymerase and modulate gene transcription. In E.coli, RpoS is one of these sigma factors and is present during stationary phase and under many stress conditions. In favourable growth conditions, RpoS is degraded by the ClpXP protease. RpoS recognition by the protease depends on the adaptor protein RssB, an unusual member of the widespread response regulator family. It has been shown that RssB can be phosphorylated but the role of its phosphorylation is still unclear. Under stress conditions, RpoS degradation is prevented by the titration of RssB by proteins called "anti-adaptors". Three anti-adaptor proteins (IraP, IraM and IraD), each induced under a different stress condition, have been identified in our lab. Although these proteins share a common function, they do not share sequence or predicted structural similarity and thus may use different mechanisms to protect RpoS from degradation. The aim of my project is to understand how RssB phosphorylation and interaction with anti-adaptor proteins regulate RpoS degradation. We showed that RssB mutants impaired in their ability to be phosphorylated have lost the ability to dimerize and no longer degrade RpoS, suggesting that RssB dimerization is required for function. Moreover, these RssB mutants did not interact with IraP and had lowered interaction with RpoS. However, interactions with IraD and IraM were conserved. A bacterial two-hybrid system and co-purification showed that IraP and IraD interact with the RssB N-terminal domain whereas IraM interacts with its C-terminal part. Finally, by screening a library of RssB random mutants for those unable to bind IraP, two functional regions of RssB were defined and studied in vitro and in vivo. The first region, in the RssB N-terminal domain, contains the RssB phosphorylation site, confirming the involvement of phosphorylation in IraP interaction. The second region, in the RssB C-terminal, is probably involved in allosteric changes allowing RssB to deliver RpoS to the protease. These experiments have uncovered unexpected complexity in the ways in which the small protein anti-adaptors modulate the activity of RssB and therefore degradation of RpoS; the results are likely to be important in understanding regulation of other response regulators as well.

NCI-CCR
GENEST, OLIVIER
Postdoctoral Fellow
Molecular Biology - Prokaryotic
Hsp90 from E. coli collaborates with the DnaK chaperone system in client protein remodeling

Molecular chaperones are proteins that assist the folding, unfolding and reactivation of other proteins. In eukaryotes, members of the Hsp90 (heat shock protein 90) family are essential ATP-dependent molecular chaperones that, with the assistance of many cochaperones, remodel and activate hundreds of client proteins including receptors, protein kinases and transcription factors. Moreover, human Hsp90 stabilizes and/or activates oncogenic proteins in cancer cells. Prokaryotic Hsp90 is highly homologous to eukaryotic Hsp90, however, its function has been elusive. The aim of this research is to elucidate the mechanism of protein remodeling by Hsp90. Studying the prokaryotic Hsp90 protein will lead to a clearer understanding of the eukaryotic chaperone. To explore the mechanism of E. coli Hsp90 (Hsp90Ec) activity, we developed an in vitro protein remodeling assay. In this assay luciferase, a model substrate, is first inactivated, and then the reactivation of luciferase is monitored over time following the addition of purified chaperone proteins and ATP. We found that reactivation of luciferase requires both Hsp90Ec and the prokaryotic Hsp70 chaperone system, known as the DnaK system and comprised of DnaK and two cochaperones. Using Hsp90Ec mutant proteins defective in ATP hydrolysis and an Hsp90 ATPase inhibitor, geldanamycin, we showed that ATP hydrolysis by Hsp90Ec was essential for protein reactivation in vitro. These results demonstrate for the first time that Hsp90Ec is a bona fide molecular chaperone, able to remodel client proteins in an ATP-dependent reaction. To explore the synergistic action of Hsp90Ec and the DnaK system in client protein remodeling, we tested if one chaperone acts before and independently of the other. Our results from staged luciferase reactivation reactions in vitro showed that the DnaK system acts first on inactive luciferase, and then Hsp90Ec and the DnaK system act together to complete the reactivation process. We also performed in vivo two-hybrid experiments and in vitro protein binding assays that showed Hsp90Ec and DnaK directly interact. These experiments provided additional evidence for the functional interaction between Hsp90Ec and the DnaK system. This study demonstrates that Hsp90Ec has chaperone activity. Moreover, it provides a much needed model system to elucidate the ATP-dependent chaperone action of Hsp90 proteins in collaboration with the DnaK/Hsp70 chaperone system.

NICHD
Waters, Lauren
Postdoctoral Fellow
Molecular Biology - Prokaryotic
Expanding the Manganese Regulon in Escherichia coli
Manganese is an essential micronutrient for organisms from bacteria to humans, serving as an enzyme cofactor and protecting cells against oxidative stress. Yet manganese is toxic in excess. Therefore, cells must carefully regulate manganese homeostasis. In bacteria, the response to changing manganese levels is controlled by the manganese-sensing transcription factor MntR. Before this work, the only gene in E. coli known to respond to manganese via MntR was mntH, which encodes a manganese importer. In this study, we identify two new genes in E. coli, mntS and yebN, that participate in manganese metabolism and propose mechanisms for their function. We unexpectedly found a new, unannotated 42-amino acid protein involved in manganese homeostasis when we undertook to characterize a small RNA of unknown function. Since this gene was transcribed divergently from mntR, we named it mntS. We demonstrated that mntS is repressed by manganese through MntR. We also showed that overexpression of MntS causes sensitivity to manganese, but not other metals. In addition, deletion of mntS perturbs proper repression of mntH by manganese, confirming that mntS alters manganese homeostasis. We found that an epitope-tagged version MntS is retained on manganese beads, while another tagged small protein is not. We therefore hypothesize that MntS may serve as a manganese chaperone to deliver the metal to Mn-using enzymes under conditions of manganese limitation. To test this, we are identifying the proteins that interact with MntS by affinity co-purification. We are also identifying the critical amino acids for MntS function by making site-directed mutants of MntS predicted

to disrupt manganese binding. We used microarray analysis of cells lacking mntR to further expand the manganese regulon and identified yebN as the first gene positively regulated by MntR in Enterobacteria. Deletion of yebN leads to profound manganese sensitivity, but does not confer a growth defect with any other metal. Since yebN is homologous to other efflux pumps, we propose that YebN may be a novel manganese-specific exporter. To test this, we are investigating the metal content of wild-type vs. yebN strains using ICP-MS. The yebN gene also possess a riboswitch element that we hypothesize may respond to manganese or other metals. We will examine the regulatory role of the riboswitch in vivo using fusions of the 5' UTR with lacZ and in vitro using RNA structure probing techniques.

NICHD
Yin, Xuefeng
Doctoral Candidate
Molecular Biology - Prokaryotic
A small membrane protein that connects two cell signaling pathways in Escherichia coli
Small proteins of less than 50 amino acids are largely understudied in all organisms. However, the few that have been characterized undertake active roles in diverse cellular processes. In particular, several prokaryotic small membrane proteins are known to regulate membrane kinases mediated cell signaling. We suggest that one conserved E. coli small membrane protein of unknown function, YneM, could play a similar role in regulating phosphate metabolism. We found by microarray analysis and confirmed by reporter gene assays that overexpression of YneM leads to rapid induction of PhoRB regulated phosphate starvation response pathway. YneM dependent activation of PhoRB regulon requires both the PhoR kinase and the downstream PhoB transcription factor as deletion of either gene abolishes this effect. It has been shown previously that expression of YneM itself is controlled by the PhoQP pathway which responds to magnesium starvation and cationic antimicrobial peptides. Therefore YneM connects PhoRB activation with PhoQP induction. For the two other examples of small membrane proteins that modulate membrane kinases, the small protein modulates activity of kinase directly. Our results from pull-down and two-hybrid assays, however, do not support a model of direct interaction between YneM and PhoR kinase. Also, we detected a partner protein that crosslinks to wild-type YneM, but not to a non-functional mutant which has a single amino acid substitution. This partner protein can still be detected in a phoR null mutant, further indicating that YneM acts on PhoRB indirectly, possibly through another partner protein involved in phosphate uptake or metabolism. To test this hypothesis, we deleted each of the three known phosphate transporters in E.coli and found that deletion of pitA, which encodes a low affinity metal phosphate transporter, abolishes the PhoRB induction upon YneM overexpression. In addition, the crosslinked band also disappears in the pitA null mutant. We are currently examining if YneM physically associates with PitA by performing pull-down experiments. It appears likely that YneM binds to and alters the activity of PitA, leading to a decrease in intracellular phosphate levels which subsequently induces PhoRB. If true, this will provide another example of small proteins that act on larger protein targets and conceivably can be used to develop artificial activators/inhibitors.

NIEHS
GAO, HUIMING
Research Fellow
Neuroimmunology
HMGB1 (high-mobility group box 1) acts on microglia Mac1 (macrophage antigen complex 1) to mediate chronic neuroinflammation that drives progressive neurodegeneration
What drives the gradual degeneration of dopamine neurons in Parkinson's disease (PD), the second most common neurodegenerative disease, remains elusive. Here, we demonstrated, for the first time,

that persistent neuroinflammation was indispensible for such neurodegenerative process. 1-methyl-4-phenylpyridinium, lipopolysaccharide (LPS), and rotenone, three toxins often used to create PD models, produced acute but nonprogressive neurotoxicity in neuron-enriched cultures. In the presence of microglia (brain immune cells), these toxins induced progressive dopaminergic neurodegeneration. More importantly, such neurodegeneration was prevented by removing activated microglia. Collectively, chronic neuroinflammation may be a driving force of progressive dopaminergic neurodegeneration. On the other hand, ongoing neurodegeneration sustained microglial activation. Microglial activation persisted only in the presence of neuronal damage in LPS-treated neuron-glia cultures, but not in LPS-treated mixed-glia cultures. Thus, activated microglia and damaged neurons formed a vicious cycle mediating chronic, progressive neurodegeneration. Mechanistic studies indicated that HMGB1 (high-mobility group box 1), released from inflamed microglia and/or degenerating neurons, bound to microglial Mac1 (macrophage antigen complex 1) and activated NF-?B pathway and NADPH oxidase to stimulate production of multiple inflammatory and neurotoxic factors. The treatment of microglia with HMGB1 led to membrane translocation of p47 (a cytosolic subunit of NADPH oxidase) and consequent superoxide release, which required the presence of Mac1. Neutralization of HMGB1 and genetic ablation of Mac1 and gp91phox (the catalytic submit of NADPH oxidase) blocked the progressive neurodegeneration. Our findings indicated that HMGB1-Mac1-NADPH oxidase signaling axis bridged chronic neuroinflammation and progressive dopaminergic neurodegeneration, thus identifying a mechanistic basis for chronic PD progression.

NINDS
Nayak, Debasis
Visiting Fellow
Neuroimmunology
Dynamics of an innate myeloid cell response to acute CNS viral infection
Viral infection of central nervous system (CNS) can have serious consequences and is a common cause of disease (e.g. meningitis, meningoencephalitis, encephalitis) worldwide. The CNS is equipped with an elaborate network of innate immune sentinels that routinely serve as first responders to an invading viral pathogen. The meninges and perivascular spaces are inhabited by specialized macrophages, and the brain parenchyma contains microglia – the most abundant CNS myeloid cell. To gain novel insights into how myeloid cells respond to an acute CNS viral infection, we utilized genomics and two-photon imaging approaches to study a pure innate response to lymphocytic choriomeningitis virus (LCMV) infection. LCMV is a noncytopathic arenavirus that causes aseptic meningitis in human and is maintained in nature by establishing persistence in mice. Intracerebral infection of mice with LCMV is a well established animal model to induce and study fatal meningitis, which is mediated primarily by LCMV specific cytotoxic T cells (CTL) and brain infiltrating myelomonocytic cells (monocytes and neutrophils). Importantly, mice lacking LCMV specific T cells do not develop meningitis, but instead become lifelong viral carriers with LCMV distributed throughout all tissues, including the brain. T cell deficient mice therefore serve as excellent model to study pure innate immune responses in the brain over time. Using intravital laser scanning two-photon microscopy (TPSLM), we studied the dynamics of green fluorescent protein labeled microglia in the living brain of LCMV infected mice unable to mount a T cell response. The viral distribution within the brain was monitored by using an LCMV recombinant expressing DsRed. Following LCMV infection, microglia (but not peripheral myeloid cells) responded robustly in the brain by proliferating. TPLSM studies also revealed that microglia reduce process complexity in regions infected by LCMV and appear to physically sequester antigen in order to prevent viral spread within the nervous system. These imaging data suggest a localized response to the presence of viral antigen. However, at the genomic level, acute infection triggered massive changes in CNS gene expression with the majority of genes being linked to innate viral control and the type I interferon system. These data

suggest that localized detection of virus by CNS myeloid sentinels can translate into engagement of an extensive anti-viral program.

NIDA
Fanous, Sanya
Postdoctoral Fellow
Neuropharmacology and Neurochemistry
Role of the prefrontal cortex in persistent cue-induced heroin craving and identification of differential gene expression in activated prefrontal cortex neurons using fluorescent-activated cell sorting (FACS)
Cue-induced heroin craving persists long after drug withdrawal and suggests equally persistent alterations of the circuits mediating this behavior. In this study, male Sprague-Dawley rats were trained to self-administer heroin, then tested for cue-induced lever-pressing during extinction tests which we interpret as an indicator of drug craving. Control rats remained in their home cages without extinction tests. Cue-induced lever-pressing was similar after 2 or 4 weeks withdrawal and associated with expression of the neural activity marker Fos in sparsely distributed sets of neurons in the medial prefrontal cortex (mPFC) and orbitofrontal cortex (OFC). To determine the role of mPFC or OFC activation in persistent cue-induced heroin craving, separate groups of rats were implanted with cannulae directed at the dorsal or ventral mPFC, or OFC, and trained to self-administer heroin. Two weeks after withdrawal, rats were infused intracranially with muscimol/baclofen (GABAA and GABAB agonists) or vehicle immediately before cue testing. Muscimol/baclofen infusions in the dorsal mPFC or OFC reduced cue-induced heroin craving, indicating that these regions are critical for the behavior. Additionally, we used fluorescence-activated cell sorting (FACS) to characterize gene expression in Fos-expressing neurons of the mPFC and OFC activated by heroin cue exposure. We hypothesized that gene expression within these neurons would be different from those in surrounding non-activated neurons. Rats were sacrificed after 90 minute extinction tests either 2 or 4 weeks after heroin self-administration training. PFC neurons, including the mPFC and OFC, were dissociated and immunolabeled for Fos and the neuron-specific protein NeuN. FACS was then used to separate Fos-labeled neurons from non-Fos-labeled neurons from the cue-exposed rats. Real-time PCR revealed that mRNAs for the plasticity-associated immediate early genes fosB, arc, egr1, and egr2 were elevated in Fos-labeled neurons, but not in non-Fos-labeled neurons, relative to all neurons from the no-test control rats. Npy and map2k6 mRNAs were also elevated in the Fos-labeled neurons, suggesting activation of neuropeptide Y-containing GABA interneurons and the p38 signaling pathway. This indicates that in rats re-exposed to heroin-related cues during late withdrawal, activated neurons undergo different molecular responses than the majority of non-activated neurons and may influence persistent cue-induced heroin craving.

NIAAA
Schank, Jesse
Postdoctoral Fellow
Neuropharmacology and Neurochemistry
The neurokinin-1 receptor antagonist L822429 suppresses stress-induced reinstatement and escalated alcohol consumption in rats
The neurokinin-1 receptor (NK1R) serves as the preferred target of the neuroactive peptide substance P (SP). The SP/NK1 system has been shown by our lab and others to be involved in alcohol related behaviors. Specifically, we have shown that mice with a genetic deletion of the NK1R consume less alcohol than controls, and this effect is mimicked by administration of NK1R antagonists to wild type mice. In clinical studies, we have found that an NK1R antagonist alleviates alcohol craving in alcoholics during early abstinence. In the studies outlined below we used L822429, an NK1R antagonist engineered to have a high affinity for the rat NK1R, to examine the role of the SP/NK1 system in operant self-

administration of alcohol, reinstatement of alcohol seeking, and escalated consumption in an alcohol preferring rat line. We found that L822429 had no effect on baseline alcohol self-administration or cue-induced reinstatement of alcohol seeking, but dose-dependently blocked the expression of stress-induced reinstatement. In a separate experiment, we measured c-fos protein expression during stress-induced reinstatement and found that L822429 blocked the stress-induced increase in c-fos in elements of the circuitry known to regulate stress-induced relapse behavior, particularly the dorsal raphe nucleus and Nucleus Accumbens shell. Given the activity profile of other anti-stress drugs, such as corticotropin releasing hormone receptor type 1 antagonists, we hypothesized that NK1R antagonists may have a more potent effect on self-administration in models of escalated consumption. To assess this possibility we used the alcohol preferring P rat, a strain that has been used extensively as a model of genetic predisposition to excessive alcohol consumption. We found that escalated alcohol self-administration in the P rat was reversed by pretreatment with L822429, decreasing responding rates to that of Wistar controls. These results suggest that the SP/NK1 system plays a significant role in excessive alcohol consumption, and further indicate the NK1R as a particularly useful target for the development of medications to treat alcoholism. Ongoing experiments explore differences in NK1R and SP expression in Wistar and P rat brain, which may underlie the differential effect of NK1R antagonists on alcohol consumption in these strains. We will also use intracranial injections of L822429 during stress-induced reinstatement to further explore the specific site of action.

NIAAA
Xiong, Wei
Visiting Fellow
Neuropharmacology and Neurochemistry
Cannabinoid potentiation of glycine receptors contributes to cannabis-induced analgesia
Cannabis attracts broad scientific interest because it produces both beneficial and harmful effects on human health. The primary psychoactive ingredient in cannabis, delta-9-tetrahydrocannabinol (THC), activates endogenous cannabinoid type 1 receptors (CB1), thereby producing some unwanted effects on human health. While most THC-induced central effects are mediated through the activation of CB1 receptors, recent studies have shown that THC and other cannabinoids can increase the activity of native and recombinant glycine receptors (GlyRs) through a CB1/CB2-independent mechanism, suggesting that the GlyRs may be an important target for cannabis in the central and peripheral nervous systems. However, little is known about the mechanisms and behavioral implication of cannabinoid-GlyR interaction. To address these questions, we conducted following experiments using various approaches involving electrophysiological recording, mutagenesis analysis, NMR spectroscopic analysis of the purified transmembrane domain of a GlyR subunit, chemical modification of THC, radioligand binding and several behavioral tests including the tail flick reflex and hot plate. Our electrophysiological recording results showed that THC, even at relatively low concentrations, enhanced glycine current (Igly) in cultured spinal neurons and HEK-293 cells transfected with GlyRs. Using mutagenesis and NMR analysis we have identified that S296 in the third transmembrane domain (TM3) of GlyR is critical for THC-induced potentiation. The polarity of the amino acid residue at 296 and the hydroxyl groups of THC is positively correlated with the magnitude of THC potentiation, suggesting a hydrogen-bond interaction between cannabinoid and GlyRs. Consistent with this hypothesis, removal of hydroxyl groups of THC results in a new compound (Di-desoxy-THC) that does not affect Igly when applied alone but selectively antagonizes cannabinoid-induced potentiating effect on Igly and analgesic effect in a tail-flick test in mice. Moreover, the cannabinoid-induced analgesia is absent in mice lacking a3GlyRs, but not in those lacking CB1 and CB2 receptors. Collectively, these findings have explored the molecular basis of THC potentiation of GlyRs and the role of GlyRs in some of cannabis-induced behaviors. The novel

mechanism underlying THC potentiation and certain types of the cannabinoid-induced analgesia can potentially lead to a strategy for the development of a new class of analgesic agents.

NINDS
CHEN, YANMIN
Postdoctoral Fellow
Neuroscience - Cellular and Molecular
Axonal Mitochondrial Transport Is Controlled by Activity-Dependent Interplay between Kinesin-1 Motor Complexes and Syntaphilin
The coordination of mitochondrial mobility is crucial for proper neuronal development and synaptic function. Motile mitochondria are recruited to the stationary pool near synapses in response to elevated cytosolic Ca2+ and synaptic activity. The mechanisms as to how motile mitochondria are recruited to stationary pool in response to neuronal activity remain unknown. Previous study in our lab identified syntaphilin (SNPH) as a mitochondrial docking receptor. KIF5 motor adaptor Miro was also reported as a calcium sensor arresting mitochondrial movement. In the current study, using snph KO mouse combined with time-lapse imaging we sought to address whether SNPH is required in Ca2+-dependent arresting of axonal mitochondrial movement. Elevated cytosolic Ca2+ in axons by treating snph (+/+) neuron with high potassium or brief electric field stimulation significantly decreased the axonal mitochondrial motility. In contrast, the same treatment of snph (-/-) neurons failed to affect axonal mitochondria motility. This result suggests that SNPH is required for Ca2+-dependent immobilization of axonal mitochondria. Using GST pull-down and immunoprecipitation of brain homogenates, we identified KIF5 motor as a specific binding partner of SNPH. Furthermore, we found that SNPH 381-466 was the binding domain for KIF5, and deleting this domain impaired SNPH-mediated mitochondrial docking. Electric field stimulation failed to arrest axonal mitochondrial transport in neurons expressing SNPH 381-466, a KIF-binding domain which interferes with the SNPH-KIF5 interaction. These results suggest that the interplay of SNPH and KIF5 is necessary for Ca2+-dependent immobilization of axonal mitochondria. SNPH neither affected the KIF5 microtubule-binding activity nor the KIF5 ATPase rate, however SNPH could disrupt the interaction between KIF5 and it's mitochondria adaptor Trak2. These results suggest that the molecular interplay between mitochondrial docking protein SNPH and transport machinery KIF5-Trak2-Miro1 is necessary for Ca2+-dependent arresting of axonal mitochondrial movement. Altogether, our study provides mechanistic insight into the molecular interplay between KIF5 motors and mitochondrial docking receptors that efficiently control axonal mitochondrial mobility in response to changes in axonal and synaptic activity.

NINDS
Cheng, Ning
Visiting Fellow
Neuroscience - Cellular and Molecular
Olfactory Model of Alzheimer's Disease Reveals Functional Recovery of APP-induced Synaptic Deficits
Synaptic loss is one of the strongest correlates to the cognitive impairment in Alzheimer's disease (AD), which involves the amyloid precursor protein (APP). Unfortunately, in most transgenic models synaptic deficits in cortical and hippocampal neurons occur slowly and involve multiple transgenes, making it difficult to study the potential recovery process. As a result, little is known if recovery of synaptic function can occur and to what extent. To address this question, we recently established an in vivo olfactory model where degeneration of olfactory sensory neurons (OSNs) can be rapidly induced by overexpressing APP alone using the tetracycline-transactivation system. We hypothesized that this model offers a unique opportunity to study APP-induced synaptic dysfunction and recovery because APP expression can be temporally controlled. In addition, since OSN axons form synapses in remarkably

precise locations in the brain called glomeruli and OSNs regenerate continuously throughout life, this model provides an organizational framework to measure circuit disruption and restoration. We showed that in 3-week old mutants, glomerular structure was greatly altered and synaptic protein expression reduced. We also observed numerous degenerating OSN axons using electron microscopy. In addition to these structural changes, in vivo functional imaging showed that synaptic release from OSN terminals was drastically reduced and behavior assays revealed a significant odor detection deficit in the mutants. Thus, our model can clearly produce rapid and severe APP-induced synaptic dysfunction. To determine if recovery was possible, we turned-off APP expression by feeding 3-week old mutants doxcycline-containing chow. We found that while 1 week of dox treatment did not significantly restore OSN numbers, glomerular structure and synaptic protein expression greatly recovered along with improved performance in the behavior assay. Thus, our data showed that in a regenerating system, functional recovery could occur even when severe synaptic deficits already exist, supporting regenerative strategies such as stem cell transplantation. Importantly, we showed that synaptic recovery preceded population restoration, suggesting synaptic function is very sensitive to APP levels and thus may be key in determining neuron survival. Together, these data present a unique model to study APP-induced synaptic dysfunction and recovery, as well as the mechanisms that regulate synaptogenesis.

NINDS
Kwon, Deborah
Doctoral Candidate
Neuroscience - Cellular and Molecular
Targeting survival motor neuron (SMN) protein degradation as a treatment for spinal muscular atrophy (SMA)
SMA is a severe neuromuscular disorder and one of the leading inherited causes of infant mortality. There is currently no treatment for SMA, which is caused by reduced levels of the SMN protein. A promising therapeutic strategy for SMA involves increasing levels of existing functional SMN protein. Our lab and others have shown that SMN is a target of the ubiquitin proteasome system (UPS). The goal of our current study is to characterize the role of the UPS in SMN protein degradation and to determine whether inhibiting the proteasome can increase SMN and ameliorate the disease phenotype in SMA model mice. We found that SMN is increased in cultured cells when the UPS is inhibited by treatment with bortezomib, an FDA-approved proteasome inhibitor. In SMA mice, bortezomib increased SMN levels only in peripheral tissues, due to its inability to cross the blood brain barrier. Bortezomib-treated animals had improved motor function, reduced spinal cord and muscle pathology and improved neuromuscular junctions, but no change in survival. Treating mice with both bortezomib and the histone deacetylase inhibitor trichostatin A resulted in a synergistic increase in SMN protein levels and extends survival of SMA mice more than trichostatin A alone. Our results demonstrate that a combined regimen of drugs that decrease SMN protein degradation and increase SMN gene transcription synergistically increases SMN levels and improves the lifespan of SMA model mice. Moreover, this study indicates that while increasing SMN levels in the central nervous system is important to extending survival, peripheral tissues may also be targeted to improve the SMA disease phenotype. Given the crucial role of the proteasome in cellular homoeostasis we are also characterizing the regulatory events upstream of the proteasome in order to identify more specific targets for therapeutic intervention. We have found that the E3 ubiquitin ligase, Mind bomb 1 (Mib1), binds and ubiquitinates the SMN protein and targets it for proteasomal degradation. Reducing levels of Mib1 using specific siRNA and miRNA increased SMN protein levels in cultured cells. These results demonstrate that SMN is a substrate for Mib1 and implicate Mib1 as a possible target for treatment of SMA.

NINDS

Poleg-Polsky, Alon
Postdoctoral Fellow
Neuroscience - Cellular and Molecular
Computer simulation of intrinsic and network mechanisms of direction selectivity is starburst amacrine neurons
The starburst amacrine cells compute direction selective (DS) signals in the retina. These cells form a unique neural network in which connection between neurons is not provided by digital axonal transmission but rather by analog transmitter release from the dendrites. Accordingly, the conventional network analysis is not applicable to these cells and the exact mechanism of DS is unclear. Experimental evidence revealed extensive inhibitory connectivity between starburst cells which according to a number of modeling studies can underlie DS computation. However, recent studies that examined responses in single starburst cells, using specially crafted light stimulation paradigms, failed to detect a significant role for network interactions during directionally presented light and one study even raised doubts on the existence of functional synaptic connections between starburst cells. To understand these conflicting findings we simulated a detailed model of a retina that included photoreceptors, horizontal and bipolar cells and a fully reconstructed small starburst cells network. We found that interactions between cells in the starburst network were readily apparent in our simulation and were important in generation of DS responses. To understand why some experimental data found an opposite effect, we simulated the experimental setups used in prior reports. This simulation revealed that small differences in the experimental setup could profoundly change the responses from the recorded cells. First, the increase in light intensity was correlated with higher excitatory input to the cell, which in turn could mask the inhibitory inputs up to a level when inhibitory starburst network interactions became undetectable. More importantly, we discovered that the light stimulation patterns used for probing DS biased the input to starburst cells. Coincidentally, these light patterns generated a larger presynaptic activity for stimuli that were supposed to be in the preferred direction for starburst activation, misleading the researchers to regard this presynaptic input as the DS response of the starburst cell. This significantly influenced their conclusions, as the bias signal was not dependent on interactions between starburst cells. Our simulation therefore not only solves the discrepancy between the experimental reports but also provides a detailed account on the workings of one of the main circuitries in the retina.

NICHD
Sukumaran, Madhav
Doctoral Candidate
Neuroscience - Cellular and Molecular
Structural and biophysical studies of the AMPA receptor N-terminal domain reveals a complex assembly-organizing function and uncovers a new neurological drug target
Neuronal network communication relies on excitatory synaptic transmission principally mediated by AMPA-type glutamate-gated ion channels (AMPARs). Native AMPARs are heterotetrameric combinations of GluA1-4 subunits with different stoichiometries conferring distinct properties (eg. exclusion of GluA2 allows Ca2+ permeation). Importantly, misregulation of heterotetramer assembly promotes excitotoxicity and is implicated in seizure and stroke. Thus, regulated AMPAR assembly critically dictates synaptic function to influence network computation. While precise mechanisms controlling AMPAR formation remain unknown, evidence implicates extracellular N-terminal domains (NTD) in directing assembly. Thus, we used analytical ultracentrifugation with fluorescence detection to directly measure heteromeric associations of GluA1-4 NTDs and x-ray crystallography to map assembly interfaces. Suprisingly, NTDs exhibited a spectrum of affinities spanning 4 orders of magnitude (Kds from <1 nM to >1000 nM) with GluA2 heteromeric Kds tighter than homomeric Kds yielding preferential heteromeric incorporation of the GluA2 subunit. Structure-guided mutagenesis of the assembly

interface bidirectionally perturbed heteromerization capacity and crystal structures of wt and mutant NTDs uncovered molecular rearrangements underlying differential assembly capacities. These findings revealed why heteromers predominate, mapped heteromerization capacity to specific interface hotspots, and also indicated physiological and pathological conditions that promote GluA2-lacking homomer formation. Moreover, GluA3 crystal structures revealed novel AMPAR NTD dynamics: a more 'open,' unconstrained conformation than GluA2, and a labile dimerization interface accommodating multiple quaternary arrangements and sliding motions of individual monomers. Computational analysis indicated two dominant modes of NTD motion: intraprotomer clamshell-motions and interprotomer counter-rotations. Re-analysis of previously solved GluA2 structures also revealed dynamic loci; however, isolated GluA2 segments were capable of large-scale translations and rotations, rather than the whole-protomer as in GluA3. Interestingly, all NTD motions could be transmitted allosterically to the rest of the channel. Together, our data support a dynamic role for NTDs in the biogenesis of functionally diverse AMPARs and widen the allosteric landscape of the receptor providing novel potential targets for neurological drug development.

NICHD
WAKE, Hiroaki
Visiting Fellow
Neuroscience - Cellular and Molecular
Activity-dependent modulation of myelin formation
• Communication between glial cells and axons in the developing adult nervous system is crucial to nervous system function. Rapid conduction of electrical impulses in the vertebrate brain depends on myelin, a multilayered membrane of insulation wrapped around nerve axons by oligodendrocytes. Myelin formation is essential for information processing because myelin increases conduction velocity at least 50 times. Stimulating myelination as a result of impulse activity in axons could enable myelin to be regulated by environmental experience, which could contribute to information processing and learning in the brain. We have shown that oligodendrocytes, the myelinating glia of the CNS, exhibit elevated Ca2+ responses in their fine processes and cell soma, in response to action-potential (AP) firing in axons. These Ca2+ responses in oligodendrocytes exhibit differential kinetics, reflective of a specific intracellular response to electrical-activity evoked neurotransmitter release from axons. Inhibition of the Ca2+ responses in oligodendrocyte processes significantly inhibits myelin formation without affecting oligodendrocyte differentiation. We have demonstrated that elevated Ca2+ responses in oligodendrocyte processes promotes the turnover of cholesterol rich domains-a possible connection site between oligodendrocytes and axons, as visualized by a pH sensitive GFP fused with transferrin receptor. Additionally, we have visualized myelin basic protein (MBP) local translation using a photo-convertible GFP fused to the 3'UTR of MBP. Using this system we have shown that de novo translation of MBP, the major constituent of myelin sheaths, occurs in direct response to axons firing AP. We also demonstrate that local translation of MBP mRNA in oligodendrocyte processes takes place at the site of the connection between oligodendrocytes and axons and is initial site of myelin formation. This is the first clear evidence that the initial steps in myelin formation are controlled by a very specific cell to cell connection, which itself depends on AP firing. Impairment of myelin regulation by aberrant AP firing can affect sensory processing and may result in visual and auditory hallucinations which are the main symptoms of schizophrenia.

NICHD
Erkkila, Brian
Postdoctoral Fellow
Neuroscience - General

Hippocampal Interneuron Migration and Integration During Development
GABAergic hippocampal interneurons (HINs) modulate glutamatergic excitatory transmission in order to allow for efficient information processing. These neurons arise from precursors in the medial and caudal ganglionic eminences (MGE,CGE), and migrate tangentially through the cortex to their final positions in the hippocampus (HIP). In order to investigate HIN migration we made use of four transgenic mouse lines which express GFP in specific cohorts of HINs. The Nkx2-1GFP mouse line selectively labels HINs coming from the MGE, while the GAD65-GFP line labels HINs derived from the CGE. Using these animals we found that HINs from the MGE and CGE each migrate in two streams, the medial and subventricular streams, first reaching the HIP around E14. Influx of HINs from both regions occurs E15-E18, with peak density of interneurons from each lineage occurs at E18 and reaching 23033 and 10271 cells/mm3 for MGE- and CGE-derived GFP+ HINs respectively. Postnatally (P30) there is a rapid and significant reduction in HIN density with levels falling ~85% to 1501.4 and 3585 cells/mm3 for the CGE and MGE derived HINs respectively. While a portion of this loss in density is explained by increasing HIP volume, our data suggest a ~50% reduction in HIN number. To examine the time course of migration, we turned to Mash1:GFP and Olig2:GFP transgenic mouse lines. These lines express inducible Cre recombinase under the control of the Mash1 (CGE) or Olig2 (MGE) drivers, as well as a floxed GFP expression cassette. When timed pregnant dams (E10-E16) are given tamoxifen (Tmx), Cre is activated only in cells expressing the driver, resulting in GFP expression. Tissue was examined 2,3 or 4 days later to create "snapshots" of the migration from the MGE and CGE, and postnatally to look at the integration of labeled HINs in the mature circuit. In the Mash1 we found that CGE HINs generated on E12.5 migrate to the HIP in three days, while those born on E14.5 and E16.5 arrive within 48 hrs despite having further to travel in an enlarging HIP. Also, Tmx administration on E14 gave rise to the most GFP+ HINs suggesting that E14 is the peak of CGE HIN generation. In the Olig2 mouse we discovered that MGE HINs generated on E10 required 4 days to migrate to the hippocampus, while those born later in development (E12) made the journey in only 2 days. Our findings present a "blueprint" for the migration of HINs from their site of origin to their positions in the final circuit.

NIMH
Fukushima, Makoto
Visiting Fellow
Neuroscience - General
The temporal dynamics of auditory cortex in awake primates
In auditory perception, temporal processing of stimuli is essential. Similar to human auditory cortex, the cortex of Macaques consists of several highly interconnected sub-regions on the supratemporal plane (STP) and adjacent superior temporal gyrus (STG). The extent to which these regions dynamically encode acoustic information is not well understood. To examine this, we designed a micro-electrocorticographic (µECoG) array to optimally record field potentials from Macaque auditory cortex. Each µECoG array has 32 recording sites on a 3x7mm grid with 1mm spacing. We implanted four µECoG arrays in each of two monkeys. Three of the arrays were inserted into the lateral sulcus. The fourth array was positioned on the surface of the caudal STG. The arrays allowed us to simultaneously record auditory evoked potentials from a large part of auditory cortex with millisecond temporal resolution. We monitored the field potentials while the monkeys passively listened to auditory stimuli including pure tones from 100 Hz to 20kHz. We examined frequency tuning by looking at the high-gamma (100-140Hz) power of auditory evoked field potentials. This analysis revealed a tonotopic map that reversed at the areal boundaries, consistent with results obtained previously with fMRI and single unit recording. Taking advantage of the high-temporal resolution, we estimated when each site showed significant discrimination among different stimulus frequencies by evaluating the high-gamma power in a 10 ms moving window. In this analysis, we found that the onset time of the discrimination increased along the

caudal-to-rostral as well as the medial-to-lateral axes, consistent with the hypothesis that auditory information is serially processed in those two directions in parallel. Taking advantage of the simultaneous recordings we next evaluated the functional connectivity among the sites, in the undriven resting state, by evaluating the pair-wise coherence in high gamma power. We found that sites with similar frequency tuning when being driven had higher coherence in the undriven resting state. This suggests that the functional connectivity in the resting state reflects the tonotopic organization of auditory cortex. Overall, these results demonstrated that µECoG array recording holds great promise for advancing our understanding of auditory processing by examining the temporal dynamics of neuronal populations.

NINDS
Liu, Junjie
Research Fellow
Neuroscience - General
Somatotopic Mapping and Beyond: Distinguishing Two Types of Stimulus-evoked Responses in Primary Somatosensory Cortex
In the classical description of cortical somatotopic organization, the primary somatosensory area 3b comprises separable and non-overlapping neural domains that selectively represent discrete body parts, such as the arm, leg and trunk. However, recent electrophysiological studies reporting robust neural responses to stimuli of other digits in the domain of a single hand digit have challenged this concept. Responses beyond the primary representation domain provide new insights into the neural integration of sensory information. Here, we study the spatiotemporal nature of these neural responses in awake non-human primates using high-resolution functional MRI and T1 mapping. BOLD fMRI was performed at 7 Tesla in 7 awake marmosets. The arm (median nerve) or leg (sciatic nerve) was stimulated by means of electrical pulses (duration: 0.4ms; amplitude: 1.5mA; repetition rate: 50Hz) delivered via contact electrodes. FMRI acquisition comprised either a 3D echo-planar imaging (EPI) sequence (spatiotemporal resolution: 4s, 0.15mm in-plane, 0.45mm slice) with short (2s) or long (20s) stimulation of the left arm or leg; or a 2D EPI (1s, 0.35mm in-plane, 0.70mm slice) with alternated left and right arm stimulation with duration varied among 1, 2, 4, 8, 16s. For anatomical determination of the arm and leg domains of area 3b, T1 maps were acquired and spatially co-registered to fMRI data. FMRI responses to long-duration stimuli of the arm or leg were spatially localized within two low-T1 regions that comprise the primary domains of the arm or leg, respectively. However, responses to short-duration stimuli of the arm or leg were more widespread and strong in the high-T1 zone comprising the trunk domain and located between the two low-T1 regions. FMRI responses to arm stimulation in the high-T1 zone were brief (< 10s) and largely invariant with the stimulus duration. In contrast, in the low-T1 region comprising the arm's domain, responses to arm stimulation remained at a plateau and lasted through the duration of stimulation. Our results suggest that sustained responses in area 3b to long stimuli are localized within the classical domains, making long stimuli more suitable for somatotopic mapping. On the other hand, the transient and duration-independent responses observed in the trunk's domain to stimuli of the arm or leg corroborate recent electrophysiological studies and suggest integrative processing of neural information across large regions of area 3b.

NIMH
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Neuroscience - General
The olfactory cortex utilizes distinct neuronal ensembles to process cortical versus amygdaloid synaptic inputs

Sensory cortices integrate synaptic inputs from a variety of cortical and sub-cortical structures to form meaningful representations of the external environment. To accomplish this task, a sensory cortex must in theory be able to delineate the anatomical origin of incoming synaptic signals to determine their unique computational value. However, it is not known if and how sensory cortices perform this function. To address this issue, we studied the mouse olfactory cortex, specifically the posterior piriform cortex (pPC), because its relatively simple structure makes it ideal for investigating general principles of cortical network function. We infected the anterior piriform cortex (aPC; a source of cortical input to the pPC) or the basolateral amygdala (BLA; a source of subcortical input) with adenoassociated virus expressing channelrhodopsin-2 and recorded the evoked excitatory postsynaptic currents (EPSCs) resulting from photostimulation of each of these fibers in pyramidal cells and interneurons in pPC slices. The aPC and BLA were chosen because they send disparate types of information to the pPC, with the aPC sending synaptic inputs necessary for encoding the physical features of odorants and the BLA sending signals needed for forming emotionally-relevant associations to these odors. Using the strength of photostimulation-evoked EPSCs as a measure of functional connectivity, we found that aPC and BLA fibers activated different subsets of pPC neurons. aPC fibers evoked 2-fold larger EPSC amplitudes in Layer 2/3 (L2/3) pyramidal cells and L1 late-spiking interneurons over most other pPC interneurons. In direct contrast, BLA fibers globally excited all pPC interneurons--with a tendency for L2 late-spiking and irregular-spiking interneurons--while providing very little, if any, excitatory drive to L2/3 pyramidal cells. This result suggests that perhaps in response to emotionally-charged stimuli (e.g. fear-inducing), the BLA transiently dampens cortical olfactory processing to help focus on imminent danger. Overall, our findings show that the pPC extracts and delineates synaptic information conveyed by a cortical versus sub-cortical structure by processing their synaptic inputs through physiologically distinct microcircuit channels. This is the first demonstration of a mechanism that allows the pPC to assign computational value to incoming synaptic signals based on their anatomical origin and generate unique outputs for each distinct type of afferent input.

NIMH
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Research Fellow
Neuroscience - General
Longitudinally mapping the influence of sex and androgen signaling on the dynamics of human cortical maturation in adolescence
Humans have systematic sex-differences in brain-related behavior, cognition and pattern of mental illness risk. Many of these differences emerge during adolescence-a developmental period of intense neurostructural and endocrine change. A popular theory draws from primate data to propose that sexually dimorphic behaviors in humans are underpinned by sexually dimorphic development of underlying brain systems, which are “masculinized” by prenatal and adolescent surges in androgen signalling. However, it is not known if (i) males and females show maturational differences during childhood and adolescence within those cortical regions sub-serving cognitive and behavioral domains where sex-differences are seen, and (ii) if maturation of such cortical regions is influenced by variations in androgen signalling. We therefore carried out the first study to longitudinally fine-map sex-differences in human brain development. These findings then served as a framework for the first longitudinal assessment of sex-steroid signalling influences on brain maturation. Here, we used an automated method to measure cerebral cortical thickness from 641 longitudinally acquired structural MRI brain scans in 284 typically developing individuals (153 male) between ages 9 and 22 years to create the first ever “movies” of sexually dimorphic brain development. We show regionally specific sex-differences in development of the cerebral cortex during adolescence. Specifically, adolescent cortical maturation was delayed in males relative to females in regions crucial for impulse control, decision-

making, social cognition and language. Conversely in cortices sub-serving visuospatial cognition which favors males, women showed a relative delay in maturation. Next, by stratifying participants through molecular analysis of the androgen receptor gene, we further show that possession of an allele conferring more efficient functioning of this sex-steroid receptor is associated with “masculinization” of adolescent cortical maturation in regions known to sub-serve sexually dimorphic domains of language and visouspatial processing. Our findings extend models first established in rodents, and suggest that in humans too, sex and sex-steroids shape brain development in a spatiotemporally specific manner, within neural systems known to underpin sexually-dimorphic behaviors.

NINDS
Renvoise, Benoit
Postdoctoral Fellow
Neuroscience - General
Mouse Model for the Complicated Hereditary Spastic Paraplegias Troyer Syndrome (SPG20).
The hereditary spastic paraplegias (HSPs; SPG1-48) are a group of neurological disorders characterized by lower extremity spastic weakness due to a length-dependent distal axonopathy of corticospinal neurons. In many cases, the underlying pathogenic mechanisms are still unknown. Loss-of-function mutations in the spartin (SPG20) gene result in HSP complicated by other features. Spartin is a ubiquitous protein that harbors an MIT (contained within microtubule-interacting and trafficking molecules) domain that interacts selectively with the ESCRT-III protein Ist1. Among other functions, ESCRT-III proteins also play a crucial role in neuronal development. To identify the functions of spartin and characterize the role of its interaction with Ist1 in SPG20 (Troyer syndrome) pathogenesis, we created SPG20-/- knockout mice. Although the mice do not present a specific phenotype we observed that the number of axonal branches in primary cortical neuron cultures is significatively higher than in SPG20+/+ mice. Overexpression of the full-length spartin in SPG20-/- neurons rescues the phenotype, suggesting that spartin is an inhibitor of branch formation. Recently, it has been shown that spartin inhibits BMP (bone morphogenetic protein) signaling, a pathway crucial for normal development of neurons. Our analysis in embryonic fibroblast (MEF) cells indicates that the phosphorylation of Smad proteins, specific proteins of the BMP signaling pathway, is higher in SPG20-/- mice. We also observed a defect in cytokinesis in Mef cells from SPG20-/- mice, with the number of multinucleated cells significantly increased. This result is consistent with our previous observations in spartin siRNA-knockdown HeLa cells. A very similar defect was also observed in profilin protein knockout mice where the number of multinucleated cells is increased in knee joint bones. As SPG20 patients also present cognitive difficulties and short stature, we analyzed the bones of SPG20-/- mice and observed a significant increase in the number of multinucleated cells in knee joint bones. Finally, we observed a defect of the percentage of fat tissue, with a significant increase in number of lipid droplets (LDs). Consistent with the increase in the specific LD marker perilipin in mice, this result supports the idea that spartin plays an important role in LD formation. Taken together, our results show that SPG20-/- mouse is a useful model for studying key cellular pathogenic features of Troyer syndrome.

NIMH
Lehmann, Michael
Postdoctoral Fellow
Neuroscience - Integrative, Functional, and Cognitive
Environmental enrichment confers stress resiliency to social defeat through an Infralimbic cortex-dependent neuroanatomical pathway
Enriched environmental (EE) housing dampens stress-induced alterations in neurobiological systems, promotes adaptability, and extinguishes submissive behavioral traits developed during social defeat

stress (SD). In the present study, we hypothesized that enrichment prior to SD can confer stress resiliency and, further, that neuronal activity in the prefrontal cortex (PFC) is requisite for this resiliency. To test these hypotheses, mice were housed in EE, standard (SE), or impoverished housing (IE) and then exposed to SD. EE conferred resilience to SD as measured in several behavioral tasks. EE-housed mice expressed elevated FosB/deltaFosB immunostaining in areas associated with emotional regulation and reward processing, i.e., infralimbic, prelimbic, and anterior cingulate cortices, amygdala, and nucleus accumbens, and this expression was largely preserved in mice receiving EE followed by SD. In contrast, in SE- or IE-housed animals, SD increased maladaptive behaviors and greatly reduced FosB/deltaFosB staining in the forebrain. We tested the putative involvement of the PFC in mediating resilience by lesioning individual regions of the PFC either before or after EE housing and then exposing the mice to SD. We found that discrete lesions of the infralimbic but not prelimbic or cingulate cortex made before but not after EE abolished the behavioral resiliency to stress afforded by EE and attenuated FosB/deltaFosB expression in the accumbens and amygdala while increasing it in the paraventricular hypothalamic nucleus. These data suggest that pathological ventromedial PFC outputs to downstream limbic targets could predispose an individual to anxiety disorders in stressful situations, whereas enhanced ventromedial PFC outputs could convey stress resilience.

NINDS
Sharma, Nikhil
Postdoctoral Fellow
Neuroscience - Integrative, Functional, and Cognitive
The evolution of learning revealed by the contribution of the new and old motor cortex to skill acquisition
Introduction: Learning to use tools is central to human society. The primary motor cortex (BA4) is critical to motor learning but it is unknown whether its phylogenetically distinct subdivisions underpin tool-use; BA4a is homologous across mammals whereas BA4p emerged later during anthropoid evolution. We hypothesized that these two parts of BA4 contribute differently to the learning of a task that involves two motor skills: a peak acceleration skill that all mammals can perform; and a peak applied force skill, required by tool-using primates e.g. in nut cracking. Methods: 30 naïve right-handed subjects (16 F, mean age 27 yrs SD 6.2) were recruited. We used diffusion weighted imaging tractography based parcellation to localize areas BA4a and BA4p in vivo; in addition to the control regions dorsal premotor cortex (PMd) and ventral premotor cortex (PMv). In a factorial design, subjects learned the task under the facilitatory effects of intermittent theta burst magnetic stimulation (iTBS) of areas BA4a, BA4p or control sites (PMd, PMv or Cz). After baseline performance iTBS was delivered guided by a frameless stereotactic tool to one site. Subjects then practiced the task in four 1-minute blocks and the following day performed a single 1-minute block to assess skill. Results: Data was normalized to baseline (no difference between groups). A two way ANOVA was performed on skill (day 2) with within-subject factors of TASK (peak acceleration, peak applied force) and between-subject factor of BA4 REGION (BA4a, BA4p). There was significant effect of TASK ($F(1,20)=5.64$ p <0.05) and interaction between TASKxBA4 REGION ($F(1,20)=12.9$ p < 0.01). A double dissociation was confirmed as stimulation to BA4p enhanced peak applied force compared to peak acceleration (t-test p <0.05) whereas stimulation to BA4a enhanced peak acceleration compared to peak applied force (t-test p <0.05). In contrast to the control areas, stimulation of BA4a and BA4p significantly enhanced acquisition of the peak acceleration skill and peak applied force respectively. Conclusions: These results demonstrate a differential contribution of the two subdivisions of BA4 to motor learning. They suggest that the capacity to acquire select motor skills changed significantly with the addition of a new part of the primary motor cortex, BA4p, during primate evolution. We suggest that the evolution of BA4p enabled select primates to acquire motor skills related to tool-use.

NIMH
Shew, Woodrow
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Neuroscience - Integrative, Functional, and Cognitive
Selective and reliable ensembles of neurons underlie the local field potential in the cortex.
The cerebral cortex is a highly complex network comprised of billions of neurons. An important goal of systems neuroscience is to understand how single neurons respond to and generate the collective dynamics of the cortical network in which they are embedded. This is a difficult task because it is currently feasible to record the activities only a minuscule fraction of the neurons in the network. Therefore, to observe collective network dynamics, we rely on indirect measurement techniques. Perhaps, the most commonly and easily studied brain signal which reflects these network dynamics are the electrical fluctuations recorded from extracellular electrodes - the local field potential (LFP). Despite decades of research, the relationship between the LFP and the spiking of single neurons has remained mysterious and is often considered poorly understood. Here, we challenge this viewpoint; we show that reliable ensembles of spiking neurons underlie the LFP signal. We measured LFP and spiking activity simultaneously from 96 electrodes implanted in premotor cortex of macaque monkeys. The 96 recording sites were arranged in a 10 x 10 grid covering a 4 mm x 4 mm patch of cortex. This allowed us to observe spatial patterns of LFP. During a 30 min recording we found that certain LFP patterns tended to repeat many times. Next, we identified the neurons which showed significant modulation in firing rate around the occurrence times of the repeating LFP patterns. We were surprised to find that most neurons fired selectively for only a few 'preferred' LFP patterns. Using this knowledge, we were able to predict the firing rate of many neurons based only the LFP measurements. This finding calls for a revision of the traditional view that LFP and spikes are weakly related. This view stems from the wrong assumption that all neurons and all LFP fluctuations can be treated equally. In contrast, by accounting for significant differences among neurons and LFP patterns, we show that cortical network dynamics, as revealed by LFP, is comprised of reliable and selective spiking ensembles.

NIDA
Sutherland, Matthew
Postdoctoral Fellow
Neuroscience - Integrative, Functional, and Cognitive
Differential Amygdala Responses to Varenicline and Nicotine in Acutely-Abstinent Smokers: Implications for individualized smoking cessation treatment?
Varenicline, through its pharmacological actions on nicotinic acetylcholine receptors, is thought to reduce smoking behaviors by ameliorating abstinence-induced withdrawal (e.g., during a quit attempt) while also dampening transient, nicotine-induced effects when nicotine is available (e.g., during a relapse). The amygdala has been theoretically linked with negative affective states accompanying acute withdrawal and such negative reinforcement mechanisms are thought to propagate continued drug use. Clinical trials have shown that varenicline helps about ~45% of smokers quit while on medication, suggesting individual differences in efficacy. Does varenicline (and nicotine) reduce amygdala reactivity in all acutely abstinent smokers, or is such an effect observable in only a portion of smokers as might be suggested by clinical trials? Abstinent (>12 hrs) smokers (N=24) completed fMRI assessments on separate days in a double-blind, placebo-controlled, two-drug crossover study. Each participant underwent ~14 days of varenicline- and placebo-pill administration (PILL factor), and completed nicotine- and placebo-patch (PATCH factor) assessments after each two-week period. Participants performed a perceptual matching task to probe amygdala reactivity in response to novel facial expressions (face vs control contrast). A median-split parsed smokers into two groups (GROUP factor) depending on how varenicline impacted overall reaction time (RT): 1) 'non-responders', in whom

varenicline had no effect on RT (39 +/- 24ms: t[11] = 1.6, p = 0.1) and 2) 'responders', who showed a significant varenicline-induced RT reduction relative to placebo (-143 +/- 24ms: t[11] = -6.0, p < 0.001). Assessing the impact of study drugs on amygdala reactivity in a 3-way mixed ANOVA revealed differential effects in these subgroups (PILL x PATCH x GROUP interaction: F[1,21] = 4.4, p = 0.047). Briefly, in the responder group, both varenicline and nicotine reduced amygdala reactivity, relative to the placebo condition by 40% and 74%, respectively. However, in the non-responder group, varenicline had no effect, and nicotine actually increased amygdala reactivity by 40%. Similar interaction patterns were observed when assessing RT measures (F[1,21] = 8.2, p = 0.01). These data indicate that subgroups of smokers respond differentially to varenicline and nicotine following acute abstinence, an observation that has the potential to impact individualized-treatment matching strategies.

NINDS
Venkatakrishnan, Anusha
Other
Neuroscience - Integrative, Functional, and Cognitive
Independent component analysis (ICA) of resting brain activity reveals transient modulation of local cortical processing by transcranial direct current stimulation (tDCS)
Neuroplasticity induced by tDCS contributes to motor learning in healthy adults and has applications in neurorehabilitation following brain lesions, although the underlying mechanisms are incompletely understood. One approach to gain insight into these mechanisms is investigation of changes induced by tDCS in resting brain dynamics (RBD), which have been shown to reflect neuroplastic modulation after learning. Recently, changes in RBD after tDCS have been shown in fMRI and EEG using connectivity-based model driven analysis. However, blind, data-driven analysis of whole-head brain activity may be better to provide unbiased understanding of mechanisms influenced by tDCS. In this regard, magnetoencephalography (MEG), with its superior temporal and spatial resolution, lends itself amenable to robust, blind and data-driven analytic approaches like ICA. Objective: To characterize tDCS effects on MEG RBD using ICA and statistical clustering. Methods: Whole-head MEG was recorded pre- and up to 30 minutes (in 4 intervals) post-tDCS (anodal, cathodal) or sham over the left motor cortex (M1) in healthy right-handed adults (n=12, stimulation order balanced). Each segment (3 min) of resting data was subjected to Infomax ICA. ICA decompositions were statistically (k-means) clustered across subjects and hierarchically across time and stimulation conditions to evaluate differences between sham and real tDCS. Spectral characteristics of identified networks were compared based on 95% confidence intervals of bootstrapped distributions. Results: We found a decrease in alpha and increase in gamma band power, starting immediately and lasting up to 25 min post-stimulation in a left parieto-motor network (PMN) with tDCS relative to sham. This network transiently engaged frontal regions in the interval 15 min post-stimulation. Using this conservative analysis, we found no differences between anodal and cathodal tDCS. Conclusion: Overall, our results suggest that tDCS over M1 produces changes in RBD in a related PMN indicative of within-network enhanced localized cortical processing. Future work will include connectivity-model based analysis within this identified network to disentangle differences between anodal and cathodal tDCS. This has implications in using tDCS to augment neuroplasticity in patients with brain lesions like traumatic brain injury (TBI) and stroke to guide effective neurorehabilitation.

NICHD
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Visiting Fellow
Neuroscience - Neurodegeneration and Neurological disorders

PPT1-deficiency impairs maturation and activity of lysosomal cathepsin D contributing to INCL pathogenesis

Neuronal ceroid lipofuscinoses (NCLs) constitute a group of common (1 in 12,500 births) childhood neurodegenerative disorders known as Batten disease. Mutations in 8 different genes cause various forms of NCLs. Infantile NCL (INCL), is the most lethal among all NCLs. It is caused by mutations in the palmitoyl-protein thioesterase-1 (PPT1) gene. PPT1 is a lysosomal enzyme that catalyzes de-palmitoylation by cleaving thioester linkages in palmitoylated (s-acylated) proteins. There are at least 100 proteins that are known to be palmitoylated for membrane anchorage and function and several of these proteins have important functions in the central nervous system. While palmitoylation is a vital post-translational modification, depalmitoylation is also essential for recycling or degradation. Thus, it is proposed that PPT1-deficiency leads to abnormal lysosomal storage of s-acylated proteins, which causes INCL pathogenesis. However, the precise molecular mechanism(s) of INCL pathogenesis still remains unclear. In the present study, we tested a hypothesis that PPT1-deficiency disrupts maturation of lysosomal cathepsin D (CTSD) and impairs degradation of s-acylated proteins (constituent of ceroid). This hypothesis is supported by the fact that CTSD gene mutations cause a severe form of NCL. To test our hypothesis, we first determined the expression and processing of CTSD in normal and INCL fibroblasts, and in the brain tissues of Ppt1-knockout (Ppt1-KO) mice that mimic INCL. We found that while high level CTSD-mRNA and -protein expression occur in PPT1-deficient cells, the processing of pro-CTSD (52 kDa) to mature CTSD (31 kDa & 14 kDa) is impaired. Moreover, we found that PPT1 deficiency caused elevated lysosomal pH, most likely due to impaired dynamic palmitoylation of proton-translocating V-ATPases, essential for maintaining lysosomal acidic pH, required for activity of lysosomal proteases including CTSD. Further, immunohistochemical analysis of brain sections from Ppt1-KO mice and those of their WT littermates showed that in Ppt1-KO mice CTSD-protein is overespressed in cortical astroglial cells. Most interestingly, while the level of CTSD-protein and activity in the cytosol of these cells were significantly elevated, the lysosomal CTSD activity was markedly reduced. Our results suggest that PPT1-deficiency alters lysosomal pH, disrupts CTSD maturation and reduces CTSD-activity, which impairs degradation of s-acylated proteins causing INCL pathogenesis.

NIMH
Chiu, Chi-Tso
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Neuroscience - Neurodegeneration and Neurological disorders

Combined treatment with the mood stabilizers lithium and valproate produces multiple beneficial effects in transgenic mouse models of Huntington's disease

Huntington's disease (HD) is an inherited neurodegenerative disorder, caused by mutation on either of an individual's two copies of a gene called Huntingtin. This mutant gene encodes an abnormal form of the protein, and results in gradual neuronal death in the striatum and cerebral cortex. HD patients suffer from various cognitive, psychiatric, and physical symptoms, such as memory loss, changes in personality, and emotional deterioration, in addition to jerky and uncontrollable movements. HD is lethal; it leads to death about 15 years after the initial symptom occurs. Currently, there is no cure for this disease. Lithium has been the first-line drug used for the treatment of bipolar disorder for more than half a century. Valproate (VPA), an anticonvulsant drug, is also effective in treating this disorder. Emerging evidence suggests that lithium and VPA have broad neuroprotective and neurotrophic properties, and that these occur via inhibition of glycogen synthase kinase 3 (GSK-3) and histone deacetylases (HDACs), respectively. We treated N171-82Q and YAC128 mice, two mouse models of HD varying in genetic backgrounds and pathological progressions, with a diet containing therapeutic doses of lithium, VPA, or both. Untreated, these transgenic mice displayed a decrease in levels of GSK-3beta Ser9 phosphorylation and histone H3 acetylation in the striatum and cortex around the onset of behavioral

deficits, indicating a hyperactivity of GSK-3beta and HDACs. Using multiple well-validated behavioral tests, we found that co-treatment with lithium and VPA more effectively alleviated spontaneous locomotor deficits and depressive-like behaviors in both models of HD mice. Furthermore, co-treatment more successfully improved motor skill learning and coordination in N171-82Q mice, and suppressed anxiety-like behaviors in YAC128 mice. This combined treatment consistently inhibited GSK-3beta and HDACs, and caused a sustained elevation in striatal as well as cortical BDNF and HSP70. These proteins are neurotrophic and neuroprotective, respectively, and have been found to be decreased in the brains of HD subjects. Importantly, co-treatment markedly prolonged median survival of N171-82Q mice from 31.6 to 41.6 weeks. Given that there is presently no proven treatment for HD, our results suggest that combined treatment with lithium and VPA, two mood stabilizers with a long history of safe use in humans, may have important therapeutic potential for HD patients.

NINDS
Landoure, Guida
Visiting Fellow
Neuroscience - Neurodegeneration and Neurological disorders
A candidate gene for hereditary spastic paraplegia type 43
Hereditary spastic paraplegias (HSPs) are inherited neurological disorders characterized by progressive spasticity and weakness with an estimated prevalence of 3-10/100,000. All modes of inheritance are seen, but dominant HSPs are most common. Over 20 recessive HSPs have been described, however, only nine disease genes have been identified to date. We previously reported a family from Mali (West Africa) with two sisters affected by spastic paraplegia with weakness of the lower limbs and marked atrophy of the distal upper extremity muscles. There was no known consanguinity, but the proportion of identity by descent by SNP analysis was higher than expected (0.67-0.77), consistent with parental inbreeding. Homozygosity mapping identified a region of extended homozygosity at chromosome 19p13.11-q12 shared by the affected sisters and not by the other unaffected family members or unrelated controls. The region of interest spans 17.7 Mb, and contains about 150 annotated genes. Sequencing of candidate genes, including SPG20 and RAB3A, GDF1, FKBP8, and eCOP in the homozygous region, was negative. We performed genome-wide exome sequencing in the two affected individuals, and found five non-synonymous homozygous single nucleotide variants in four genes within the previously identified locus. One sequence variant could not be confirmed by Sanger sequencing, and three were seen in controls. The remaining variant is located in the C19orf12 gene at position c.154G>C, predicting the amino change A52P. This residue is conserved across species, and was not found in about 300 ethnically matched controls. There was no difference in patient C19orf12 mRNA levels in lymphoblastoid cells compared to unaffected family members or normal controls by qRT-PCR. However, immunostaining of transfected cells showed that the mutant C19orf12 localized to the nucleus while the wild-type was present in the cytoplasm. C19orf12 is predicted to be a membrane protein, and the A52 residue is localized in a predicted membrane helix domain. The substitution of alanine to proline appears disrupt C19orf12 a membrane targeting motif. Thus, we identified a novel gene for recessive HSP that adds to our understanding of the cellular biology and pathogenesis of this group of disorders. Additional investigations, including functional studies of the gene product, are now being undertaken to characterize the role of this protein in the health of the corticospinal tract, which degenerates in HSP.

NIA
Okun, Eitan
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Neuroscience - Neurodegeneration and Neurological disorders

Deficiency of Toll-like receptors 3 and 4 extend lifespan in the N171-82Q mouse model of Huntington's Disease

Huntington's Disease (HD) is an incurable inherited neurodegenerative disorder, in which symptoms range from motor coordination impairments to psychiatric disorders and dementia. Toll-like receptors (TLRs) are a family of innate immune receptors, widely known for their roles in detecting invading pathogens. Recent data suggest that these receptors also influence neural stem cell plasticity, neuronal cell differentiation and affective and cognitive behaviors. We crossed a mouse model of HD (the N171-82Q strain) with mice deficient for TLR2, TLR3 or TLR4 to investigate whether these TLRs participate in the pathogenesis of HD. We found that HD mice with a homozygous (HO) deficiency for TLR3 exhibit prolonged survival and a reduced rate of weight loss compared with wild-type (WT) mice or mice with a heterozygous (HT) deficiency for TLR3. Additionally, we found that HD mice with a HO but not WT or HT deficiency for TLR4 exhibit extended survival, without a significant effect on weight loss. In contrast, neither HO nor HT deficiency for TLR2 affected the survival of HD mice. These results suggest a previously unrecognized role for innate immune-related signaling pathways in the pathogenesis of HD. We are currently studying the molecular mechanism by which TLR3 and 4 deficiencies confer survival extension to HD mice; understanding the underlying molecular mechanism for this effect could help in designing novel treatments for HD.

NICHD
Sarkar, Chinmoy
Visiting Fellow
Neuroscience - Neurodegeneration and Neurological disorders

Lysosomal ceroid depletion by a small molecule: Therapeutic implications for an inherited childhood neurodegenerative storage disease

Infantile neuronal ceroid lipofuscinosis (INCL) is one of the most lethal childhood neurodegenerative storage disorders. Children afflicted with the disease are normal at birth but around 2 years of age they develop psychomotor retardation and retinal blindness. By age 4, these children fail to manifest any brain activity. They remain in a vegetative state for another 8-10 years before death. The disease is caused by mutations in the gene encoding palmitoyl-protein thioesterase-1 (PPT1). PPT1 catalyzes cleavage of thioester linkages in palmitoylated (s-acylated) proteins facilitating their recycling or degradation. Lack of PPT1 in INCL causes abnormal accumulation of palmitoylated (s-acylated) proteins (constituent of ceroid). Currently there is no effective treatment for INCL and it remains a uniformly lethal disease. It is well known that hydroxylamine (HA), a small molecule, cleaves thioester linkages in s-acylated proteins with high specificity and functionally mimics PPT1. However, toxicity precludes its clinical application although HA-derivatives may be non-toxic. In this study, we have screened several derivatives of HA based on three criteria that: (a) it is non-toxic, (b) efficiently cleaves thioester linkage in s-acylated proteins mediating depletion of ceroid and (c) possesses antioxidant property. We found that three non-toxic derivatives of HA disrupt thioester linkages in a model thioester compound, [14C]-palmitoyl-CoA. Moreover, one of these derivatives, N-t-butylhydroxylamine (NtBuHA), efficiently cleaves thioester linkages in s-acylated proteins from cultured INCL lymphoblasts and in brain tissues of Ppt1-knockout (Ppt1-KO) mice, a reliable animal model of INCL, fed with a diet containing NtBuHA. Further, NtBuHA mediated depletion of lysosomal ceroids, alleviated oxidative stress and inhibited apoptosis both in cellulo and in the brain of Ppt1-KO mice on NtBuHA diet. Most importantly, when we evaluated the NtBuHA-treated and untreated Ppt1-KO mice using rotarod test, we found significant improvement in motor function of the treated group. Our results define a novel pharmacological approach to lysosomal ceroid depletion and provide the proof of principle that small molecules such as NtBuHA may have therapeutic potential for INCL.

NIDA
Yu, Seongjin
Visiting Fellow
Neuroscience - Neurodegeneration and Neurological disorders
Suppression of endogenous PPARgamma expression increases vulnerability to metamphetamine-induced injury
Rationale: Methamphetamine (Meth) is a commonly abused drug and dopaminergic neurotoxin. Repeated administration of high doses of Meth induces programmed cell death, suppression of dopamine (DA) release, and reduction in locomotor activity. Pretreatment with Peroxisome Proliferator-Activated Receptor gamma (PPARgamma) agonists reduced Meth -mediated neurodegeneration. Objectives: The purpose of this study was to examine the role of endogenous PPARgamma in protecting against Meth toxicity. Methods: Adeno-associated virus (AAV) encoding the Cre recombinase or GFP gene was unilaterally injected into the left substantia nigra of loxP-PPARgamma or control wild type mice. The expression of PPARgamma was examined by qRTPCR and immunohistochemistry (IHC). Animals were treated with high doses of Meth 1-month after viral injection. Behavioral tests were examined using rotarod and rotometer. In vivo voltammetry was used to examine DA release/ clearance at 2 months after Meth injection. Immunoreactivity of tyrosine hydroxylase in striatum and nigra was analyzed by IHC. Results: Administration of AAV-Cre, but not AAV-GFP, selectively removed PPARgamma in left nigra in loxP-PPARgamma mice. The loxP-PPARgamma/AAV-Cre mice that received Meth showed a significant reduction in time on the rotarod and exhibited increased ipislateral rotation using a rotometer. In vivo voltammetric data indicate that the peak of DA release and the rate of DA clearance were significantly attenuated in the striatum of loxP-PPARgamma mice receiving AAV-Cre, compared to WT mice pretreated with AAV-Cre. Tyrosine hydroxylase immunoreactivity was reduced in nigra and dorsal striatum in loxP-PPARgamma/AAV-Cre mice receiving high doses of Meth. Conclusions: Our behavioral, histological and electrochemical data suggest that deficiency in endogenous PPARgamma does not alter dopaminergic function in the absence of injury, but enhances neurodegeneration after exposure to high dose Meth. It has been reported that endogenous PPARgamma expression is suppressed in certain clinical disorders and can be inhibited by various pharmacological agents. Deficiency in endogenous PPARgamma in these conditions may increase vulnerability to Meth insults and that endogenous PPARgamma may play an important role in reducing Meth toxicity in vivo. Our data supports the idea that PPARgamma is important for minimizing brain injury and may be a target for pharmacotherapy in neurodegeneration.

NICHD
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Neurotransmission and Ion Channels
Developmental synaptic NMDA receptor remodeling by Kv4.2 potassium channels in vivo.
Aberrant neural activity during development alters synaptic function underlying learning and memory and ultimately can lead to neuropsychiatric disorders such as schizophrenia and Alzheimer's disease. Thus, identifying mediators of neural activity and synaptic modification is vital to understanding normal CNS development and pathology. Early in development, excitatory synapses in the hippocampus transmit primarily through NMDARs. These synapses are functionally silent at birth but during development they become functional by inserting AMPARs. As synapses mature, NR2B-containing NMDARs are replaced by those containing NR2A subunits. These synaptic modifications require precise control of neural activity. In the hippocampal CA1 pyramidal neurons, excitability is controlled by the voltage gated potassium channel subunit, Kv4.2. We found that blockage or deletion of Kv4.2 increased the frequency and amplitude of dendritic action potentials. Also, Kv4.2 expression in cultured neurons

increased with days in vitro suggesting a link between Kv4.2 and synapse maturation. Therefore, Kv4.2 knock-out (KO) mice along with acute in vivo overexpression of Kv4.2 were used to test whether Kv4.2 expression level influences synaptic development in the hippocampus. Electrophysiology recordings in acute hippocampal slices showed that the NMDA/AMPA ratio and synaptic NR2B fraction were higher in both neonatal WT and adult Kv4.2 KO than in adult WT. Acute overexpression of Kv4.2 by injecting Sindbis virus carrying EGFP-tagged Kv4.2 into the hippocampus lowered the NMDA/AMPA ratio and NR2B fraction in infected neurons compared to uninfected neurons in both neonatal WT and adult Kv4.2 KO. These results indicate for the first time that developmental synaptic modifications depend on Kv4.2 expression level. To see if Kv4.2 KO delays synaptic maturation, silent synapses were measured by a minimal stimulation protocol. In neonatal Kv4.2 and WT neurons, silent synapses contribute 50% of total synapses. Surprisingly, neurons in adult Kv4.2 KO also were found to contain a large number (55%) of silent synapses while only 2% of synapses were silent in adult WT. Importantly, overexpression of Kv4.2 in neonatal WT reduced the incidence of silent synapses (29%). These results indicate that downregulation of Kv4.2 during development delays synaptic maturation by altering synaptic modifications, which may underlie abnormal CNS development and neuropsychiatric disorders.

NICHD
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Neurotransmission and Ion Channels
Assembly mechanisms of heteromeric glutamate receptor ion channels
Glutamate receptor ion channels (iGluRs) mediate excitatory neurotransmission in 60% of brain synapses and play key roles in neural development, synaptic plasticity, motor function, learning and memory formation. The three major iGluR subtypes: AMPA, kainate and NMDA receptors are tetramers in which the extracellular domains assemble as a dimer of dimers. During biogenesis, appropriate members of a diverse subunit pool are selected to form assemblies of restricted stoichiometry and composition with unique functional properties. While, the molecular mechanisms regulating this assembly are largely unknown, the semiautonomous, extracellular amino terminal domains (ATD) have been shown to play a key role in the assembly process. We used analytical ultracentrifugation (AUC), X-ray crystallography and electrophysiology to study this mechanism in kainate receptors. Our studies reveal that the ATDs of kainate subtype iGluRs from different gene families, GluR6 and KA2, assemble as heterodimers in solution with a Kd of 76nM, 5400 fold lower than the Kd for KA2 homodimerization. Crystal structures of GluR6/KA2 ATD heterodimer and heterotetramer assemblies reveal the molecular basis for the high affinity and specificity of assembly. Using these structures as guide, we performed a mutant cycle analysis to probe mechanisms for heterodimer formation. The analysis yielded coupling coefficients of 0.8-1.7 suggesting that the process of heterodimer assembly is additive without strong cooperativity. Further, the heterotetramer crystal structure showed that GluR6 and KA2 ATDs assemble as pairs of heterodimers with the KA2 subunits at the lateral edges of the tetramer, while the GluR6 subunits mediate the dimer of dimers assembly on the 2-fold axis of molecular symmetry, yielding a Kd of 3.5 uM for tetramer assembly. We validated that the same ATD tetramer assembly occurs in full-length kainate receptors by cysteine mutant cross-linking experiments and electrophysiological assays with subtype selective ligands. Our results establish that ATD domains play a key role in facilitating the efficient assembly of heteromeric iGluR assemblies. The high affinity of the KA2 subunit for GluR6 ensures that ATD heterodimers will form early during the process of biogenesis, prior to trafficking, and ensures a 2:2 stoichiometry of assembly while in addition providing a mechanism to suppress formation of GluR6 homotetramers lacking the KA2 subunit.

NIEHS

chang, xiaoqing
Visiting Fellow
Pharmacology and Toxicology/Environmental Health
A Physiologically Based Pharmacokinetic Model of Micro and Nano Sized Fluorescent Polystyrene Spheres in Rats
Human exposure to engineered nanoparticles has increased dramatically over the past decade. However, their potential adverse effects on human health still remain uncertain. To assess nanotoxicities, it is important to understand the pharmacokinetics of nanopartices. Physiologically-based pharmacokinetic (PBPK) modeling is a powerful tool for understanding the unique kinetic properties of nanoparticles. The lymphatic and reticuloendothelial systems (RES) have been shown to play important roles in particle uptake and sequestration. However, none of the currently available PBPK models for nanoparticles take into account these two components. In our study, we developed a PBPK model incorporating both lymphatic system and RES with a focus on toxicokinetic comparisons between nanoparticles and chemically identical micro particles. Rats were exposed to four different sizes of fluorescent polystyrene spheres (1000, 100, 40, 20 nm) via a single intravenous (IV) injection or oral pharyngeal aspiration (PA) into the airways followed by tissue distribution assessment over 90 days. A diffusion-limited, whole-body model was developed based on these data incorporating lymphatic system, macrophage phagocytosis and plasma protein binding. Using a step down procedure, we fit the data for 1000 nm particles and used this model to predict kinetics for the smaller particles. The 1000 nm model adequately predicted tissue kinetics for the three nano sized particles in liver, lung, and heart for IV exposure. The 1000 nm model also predicted tissue kinetics well in lung, liver and gastrointestinal tract for PA exposure. However, the prediction from micro to nano failed in most of the other organs, suggesting a mixture of common and uncommon kinetic mechanisms of these particles across different organs. Through a process of elimination using statistical likelihoods, we further identified modifications to the 1000 nm model providing an adequate fit to the 100 nm data. The process was repeated at each subsequent size to identify differences in kinetics between particles. A comparison of model parameters optimized for each sized particle indicated that the RES plays a major role in determining particle distribution; and that the smaller sized particle moved more freely between blood and tissues. In summary, this work provides a general framework for elucidating the kinetics of nanoparticles and should greatly enhance our understanding of nanotoxicity.

NIEHS
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Pharmacology and Toxicology/Environmental Health
Leptin signaling synergizes environmental bromodichloromethane exposure-induced post translational protein oxidations, antigen presentation and exacerbation of steatohepatitis of obesity
Although direct exposure to high doses of disinfection byproducts like bromodichloromethane (CHBrCl2) is rare, low exposures from the environment are not uncommon especially from domestic water supply, swimming pools and ground water seepage. We tested the hypothesis that metabolic oxidative stress arising from such low exposures in obese mice synergizes with high leptin levels in these individuals and leads to exacerbation of steatohepatitis (inflammation of steatotic liver) through lipid peroxidation, post translational protein oxidations and triggering of innate immune mechanisms. Results indicated that low CHBrCl2 exposure (2mMoles/kg) caused significantly increased lipid peroxidation in hepatocytes of diet-induced obese (DIO) and leptin-knockout mice at 6h. There was also a significant increase in protein radical adducts and post-translational tyrosine nitration (from the reaction of the tyrosyl radical with nitrogen dioxide radical) in sinusoidal cells including CD68 positive Kupffer cells at 24 hrs when compared to leptin-knockout or lean control mice at 24h. The role of NADPH oxidase in free radical

damage was confirmed using apocynin, a specific inhibitor for this enzyme. To study the role of the molecular basis of leptin-induced redox signaling in CHBrCl2-exposed obese mice, isolated Kupffer cells that were trans-cultured with hepatocytes from wild-type and leptin-knockout mice were probed for TNF-alpha secretion, a hallmark of macrophage activation and antigen presentation. Results showed that Kupffer cells from DIO mice exposed to CHBrCl2 had enhanced TNF-alpha release and increased MHC Class II expression, when compared to corresponding lean controls and leptin-knockout mice. Kupffer cells co-incubated either with 4-hydroxy Tempol, a superoxide dismutase mimetic, or higher concentrations of the spin trap DMPO (for freezing the radical reaction) significantly reduced macrophage activation and MHC Class II expression. Further recombinant leptin supplementation in leptin knockout mice and using SC-409, a MAPK-P38 inhibitor identified MAPK-P38 as a downstream signaling molecule for leptin action in this model. Taken together, our data for the first time suggest that CHBrCl2 bioactivation from the environment and higher circulating leptin generates an inflammatory cascade and free radical damage in obese mice. These events can be predictable causes for exacerbation of stetaohepatitis in obesity.

NIAAA
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Visiting Fellow
Pharmacology and Toxicology/Environmental Health
ROLE OF POLY (ADP-RIBOSE) POLYMERASE 1 (PARP-1) IN LIVER INJURY, INFLAMMATION, AND FIBROSIS
Poly (ADP-ribose) polymerase 1 (PARP-1) is a nuclear enzyme which plays an important role in regulating DNA repair, cell death and modulating key inflammatory responses. Pharmacological inhibitors of PARP provide significant therapeutic benefits in multiple preclinical disease models associated with tissue injury and inflammation, and are being evaluated as promising novel treatments for cancer both in monotherapy, as well as in combination with chemotherapeutic agents and radiation, in humans. However, the role of PARP activation in the pathophysiology of liver fibrosis and steatosis is elusive. Genetic deletion of PARP-1 and/or pharmacological inhibition of the enzyme ameliorated acute and chronic liver injury in two different mice models (injury induced by acute and chronic carbon tetrachloride (CCL4) and alcohol exposure) by attenuating oxidative stress, inflammation, cell death and fibrosis. Furthermore, PARP inhibitors were able to reverse the established fibrosis caused by chronic CCl4 administration. Pharmacological inhibition or genetic deficiency of PARP-1 mitigated oxidative stress-induced hepatocyte death, Kupffer cell activation, and stellate cell proliferation in vitro. We have also shown an important role of PARP-1 in mediating alcohol-induced liver injury by using in vivo mouse models and/or in vitro cellular systems. Genetic deletion of PARP-1 and/or pharmacological inhibition attenuated liver damage, steatosis and oxidative stress caused by alcohol administration. Finally, in human cirrhotic liver samples obtained from patients infected with hepatitis B virus or suffering from alcoholic cirrhosis we observed increased PARP activity and oxidative stress similar to our animal data validating our findings. We have shown with a combined genetic and pharmacologic approach that PARP plays a major role in the pathomechanism in various liver diseases leading to cirrhosis and ultimately liver cancer. Furthermore, we provided a multi-target mechanism of action for PARP-1 inhibitors: (1) protection of hepatocytes against oxidative damage-induced necrotic cell death (2) reducing inflammation by attenuating Kupffer cell activation (3) reducing fibrosis by inhibiting stellate cell proliferation and migration. Collectively, our results demonstrate for the first time that targeting PARP can offer an excellent novel strategy for the treatment of various liver diseases associated with inflammation, steatosis and fibrosis.

NIEHS
SAITO, KOSUKE

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Pharmacology and Toxicology/Environmental Health
The role of the potassium channel KCNK1 in the sexual dimorphic centrilobular hypertrophy induced by phenobarbital in mouse liver
The liver is one of the organs that exhibit sexually dimorphic responses against therapeutics and xenobiotics. For example, the susceptibility of non-genotoxic liver tumor development caused by phenobarbital (PB) is higher in male than female mice. Although PB activates the constitutive active/androstane receptor (CAR) to cause the male-predominant tumor development, the expression of CAR and the PB-caused induction of a classical CAR target gene Cyp2b10 are sex-independent in mouse liver. Therefore, the molecular mechanism of the male-predominant tumor development is not well understood. Given the fact that hypertrophy and hyperplasia precede tumor development, we have now identified KCNK1 (an inwardly rectifying potassium ion channel) as a CAR target that may be responsible for the male-predominant development of liver tumors. First, the expression of KCNK1 was induced by PB treatment only in male but not in female liver. Second, the induction of KCNK1 was no longer observed in livers of Car-/- male mice. Promoter analysis demonstrated that CAR activated the Kcnk1 promoter through a 97-bp DNA sequence (97-bp RE). ChIP assay revealed that PB treatment enhanced the binding of CAR on the 97-bp RE only in male but not female liver. These results imply that the male-specific binding of CAR on the 97-bp RE is responsible for the male-specific activation of the Kcnk1 gene by PB treatment. In general, potassium channels are reported to play a role in regulating carcinogenesis, apoptosis, and cellular volume. Therefore, we used histological analyses to examine whether KCNK1 plays a role in the PB-induced development of liver tumors. KCNK1 was induced in the hepatocytes around the central veins but not in those around the portal veins of PB-treated male liver, while no significant induction of KCNK1 was observed in female liver. This male-specific, centrilobular hepatocyte-specific induction of KCNK1 was consistent with the incidence of the PB-induced centrilobular hypertrophy. Moreover, Kcnk1-/- male mice exhibited a significant decrease in the PB-induced centrilobular hypertrophy. Although the KCNK1-regulated hypertrophy is not directly linked to tumor development, our present experimental results suggest that KCNK1 can be a possible CAR target for sexually dimorphic non-genotoxic carcinogenesis induced by PB. In addition, our results provide an animal model for further investigations to delineate the molecular mechanism of this non-genotoxic tumor development.

NIEHS
Wang, Xueqian
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Pharmacology and Toxicology/Environmental Health
Nuclear Factor E2-Related Factor-2 (Nrf2) Regulates P-glycoprotein Expression at the Blood-Brain Barrier (BBB) by Acting Through p38 MAP Kinase
At the BBB, the ATP-driven efflux pump, P-glycoprotein (Pgp) is a major impediment to CNS pharmacotherapy. Signals that modulate Pgp transport function are complex and not fully mapped. Recent studies show that Pgp is upregulated by xenobiotics acting through nuclear receptors, e.g., aryl hydrocarbon receptor (AhR). Here we show that ligands for Nrf2 increase Pgp-mediated transport and transporter protein expression in rat brain capillaries. Nrf2 senses oxidative/electrophilic stress and induces multiple cytoprotective proteins, including antioxidant and glutathione generating enzymes, but its ability to modulate expression of transport proteins is largely unexplored. Under basal conditions, Nrf2 is retained in the cytosol. Upon activation, Nrf2 translocates to the nucleus, where it forms a heterodimer with a small Maf protein, binds to an antioxidant response element (ARE) and induces transcription. We found that the promoter region of rat Pgp contains multiple Nrf2 binding sites. We used freshly isolated rat brain capillaries, a fluorescent Pgp substrate and confocal microscopy to

monitor chages in Pgp transport activity. Exposing capillaries to the Nrf2 ligand, sulforaphane (SFN, 0.1-10 µM), a naturally occurring compound present in cruciferous vegetables, increased Pgp activity in a concentration-dependent manner. Increased transport was observed with 2 h SFN exposure and reached a maximum with 3h of exposure. Inhibiting transcription with actinomycin D or inhibiting translation with cycloheximide abolished SFN-induced upregulation of transport. Another Nrf2 activator, tert-butylhydroquinone (tBHQ), widely used in food preservatives, also significantly increased Pgp transport activity. Exposing rat brain capillaries to SFN (1-10 µM) caused a concentration-dependent increase of Pgp protein expression assayed by Western blot. Electrophoretic Mobility Shift Assay (EMSA) detected binding of Nrf2 to ARE in nuclei from rat brain capillaries exposed to SFN. Pretreament with SB203580, a p38 mitogen-activated protein kinase inhibitor, abolished SFN- and tBHQ- induced upregulation of Pgp transport, while inhibitors of MEK and PI3 kinase were without effect. These results implicate p38 signaling in Nrf2 induction of Pgp activity. Thus, the BBB is tightened selectively by dietary constituents that are Nrf2 ligands, providing increased neuroprotection but at the expense of reduced penetration of certain therapeutic drugs.

NHLBI
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Physiology
Resin-Assisted Capture Methods show that S-nitrosylation Exerts Cardioprotection During Ischemia/Reperfusion Injury by Directly Reducing Cysteine Oxidation
Redox modifications play an important role in many cellular processes, including cell death. Ischemic preconditioning (IPC) is a protective mechanism of the heart that reduces damage sustained from prolonged periods of ischemia and has been shown to involve redox signaling. S-nitrosylation (SNO) is a reversible, thiol-based redox modification that is greatly increased following myocardial IPC and is thought to play an important role in cardioprotection. Furthermore, inhibition of S-nitrosoglutathione-reductase has been shown to increase protein SNO and is also cardioprotective. SNO is thought to provide cardioprotection, in part, by modulating enzyme activity. SNO may also provide cardioprotection by reducing irreversible cysteine oxidation that occurs via the formation of reactive oxygen species (ROS) during ischemia/reperfusion (IR) injury. Irreversible oxidation has the potential to alter protein function and/or denature proteins, thus leading to a period of sustained myocardial dysfunction. Therefore, in order to test the hypothesis that SNO provides cardioprotection by directly blocking irreversible cysteine oxidation, we developed a method to identify SNO and oxidation sites using resin-assisted capture (SNO-RAC and Ox-RAC). This method also provides a relative measure of protein SNO and oxidation levels at each respective modification site. Langendorff perfused hearts were subjected to various perfusion protocols (control, IPC, IR, IPC-IR) and homogenized. Each sample was divided into two equal aliquots, and subjected to the SNO-RAC and Ox-RAC procedures in order to simultaneously analyze SNO and oxidation. With IPC, we identified 31 different SNO proteins, 27 of which showed a significant increase in SNO compared to baseline. There was also a reduction in cysteine oxidation with IPC-IR when compared to IR injury alone. Interestingly, of the proteins that showed a significant increase in SNO with IPC, 76% showed decreased oxidation or no oxidation following ischemia and early reperfusion (IPC-IR) when compared to IR alone; for non-SNO proteins, oxidation was reduced by only 50%. We further demonstrated that IPC-induced protein SNO is quickly reversible upon early reperfusion. These results support the hypothesis that SNO provides cardioprotection by shielding cysteine residues from ROS-induced oxidation during IR injury. Therefore, the level of protein SNO plays a critical role in IR injury, where ROS production is increased.

NIAAA

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Physiology
Elevating blood ketone bodies increases mitochondrial biogenesis and uncoupling protein 1 in brown adipose tissue of mice
High fat very low carbohydrate ketogenic (KG) diet has been shown to improve glycemic control in type II diabetic patients. However, the strict requirement to avoid carbohydrate intake is very difficult to comply with for long durations. Therefore, our lab has synthesized an ester of ketone body D-b-hydroxybutyrate and R-1,3 butanediol (ketone ester, KE), which when added to regular diets can increase blood ketone levels without requiring any dietary restrictions. Mice on ketogenic (KG) diet have lower weight gain and increased metabolic rate, but the precise mechanisms are not well understood. Brown adipose tissue (BAT) is an important tissue for energy homeostasis in rodents and humans, as it dissipates metabolic energy by mitochondrial uncoupling mediated through uncoupling protein 1 (UCP1). BAT activity correlates negatively with BMI, spurring an interest in identifying agonists that activate BAT. We hypothesized that increased BAT activity due to elevated blood ketones is responsible for the increased metabolic rate and lower weight gain in mice on KG diet. To prove it, C57BL/6J mice were ad-lib fed two different ketone-elevating diets- (i) a commercially available ketogenic (KG) diet containing high amounts of saturated fats, and (ii) a liquid diet containing KE. Control animals were fed NIH chow or pair-fed a liquid control diet. The KG diet increased blood D-?-hydroxybutyrate (?HB) to 1.5 mM, while the KE diet increased it 4-7 mM- levels seen in prolonged fasting in humans. Animals on the KG and KE diets had significantly lower blood glucose levels than the chow diet in both light and dark cycles, and significantly lower blood glucose levels than pair-fed liquid controls in the dark cycle-coincident with maximum ketone concentrations. Feeding the diets for 1 month caused a significant increase (~3-fold) in UCP1 as well as mitochondrial electron transport chain (ETC) proteins in the interscapular BAT, with increases from 30-300%. Levels of the mitochondrial biogenesis master-regulator PPARgamma coactivator 1 alpha (PGC-1alpha?, cAMP response element binding protein (CREB), silent information regulator (Sirt1), PPARalpha and PPARgamma were also elevated by the test diets. These results show that elevated blood ketone bodies reduce blood glucose levels and activate BAT, and ketone-elevating agents such as the KE could be used to treat obesity and the metabolic syndrome without the toxic effects associated with pharmacological PPAR agonists.

NIDDK
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Protein Structure/Structural Biology
Structural insights into the assembly of a fission machine
Clathrin-mediated endocytosis (CME) is a highly regulated pathway of fluid phase uptake that is essential in a wide array of biological processes. The efficiency of CME depends on the function of the large, multidomain GTPase dynamin, which assembles into collar-like structures at the necks of invaginated clathrin-coated pits and facilitates coated vesicle release by catalyzing fission of the plasma membrane. Assembly of purified dynamin tetramers in vitro yields helical structures comparable to the collars observed in vivo. The formation of these oligomers stimulates dynamin's basal GTP hydrolysis >100-fold. Functional studies suggest that stimulated turnover triggers disassembly of the dynamin collar during CME and promotes the subsequent membrane remodeling events that lead to fission; however, little is known about the structural interactions and conformational changes that drive these processes. We previously demonstrated that stimulated hydrolysis arises from the transition-state-dependent dimerization of dynamin's catalytic G domains and proposed that this interaction occurs between two dynamin tetramers within the context of the assembled polymer. To investigate these

interactions further, we have used cryo-EM and iterative helical real space refinement to produce an 11Å reconstruction of the assembled dynamin polymer in the presence of the non-hydrolyzable GTP analog GMPPCP. This map reveals new structural features not resolved in previous lower-resolution reconstructions. Additionally, we solved the 2.3Å structure of GMPPCP-bound GG, a minimal fusion protein that links the G domain of human dynamin 1 to its GTPase effector domain. Computational fitting of GG-GMPPCP and other crystallized dynamin fragments into our cryo-EM polymer map allowed us to localize different domains within the assembled structure and define their connectivity. Moreover, this docking model shows that G domain dimers can only form between tetramers in sequential rungs of the dynamin helix. Assembly of the helical collar thus primes the dynamin subunits for stimulated turnover. Structural comparison of GG-GMPPCP to the previously characterized GG-GDP.AlF4-transition-state complex also identifies a major hydrolysis-dependent conformational change that could facilitate dynamin detachment from the membrane surface. Finally, chemical crosslinking experiments provide new insight into the architecture of the dynamin tetramer, revealing it to be dimer of domain-swapped dimers.

NIDDK
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Protein Structure/Structural Biology
Structural Insights into Iron Pirating by Pathogenic Neisseria
Pathogenic Neisseria are Gram-negative bacteria that are responsible for gonorrhoea, meningococcal septicaemia, and bacterial meningitis. Once entering their human host, Neisseria, along with other pathogenic bacteria, must acquire iron for survival by hijacking iron from iron binding bindings such as hemoglobin and transferrin. In Neisseria, two outer membrane proteins, transferrin binding protein A (TbpA, receptor and transporter) and transferrin binding protein B (TbpB, a lipoprotein co-receptor), work in concert to bind and extract iron specifically from human transferrin (hTF), making them primary targets for both drug and vaccine development. To aid these efforts, we determined the crystal structures of (1) TbpA in complex with hTF and (2) TbpB alone, identifying key residues and extracellular loops important for therapeutic development. The binding interface between TbpA and hTF is very large having a buried surface area of ~2500 Å^2 and involves 87 residues from TbpA and 67 from hTF, with surface charge also playing a key role in the interaction. As predicted, the structure of TbpA contained an N-terminal plug domain tucked within the 22-stranded beta-barrel transmembrane domain. Interestingly, a previously reported motif important for iron import (EIEYE) was found within a highly electronegative cavity formed by the plug and barrel domains. Possibly the most exciting features of the TbpA structure are the very large extracelluar loops (11 total) which were as large as 70 residues, providing further evidence of this protein's potential for use in vaccine development. Using an ELISA assay to investigate this idea further, we show that polyclonal antibodies against Loops 3, 7, and 11 and against a loop from the N-terminal plug domain were able to effectively block hTF binding by as much as 80% and polyclonal antibodies against full length TbpA could completely block hTF binding. Furthermore, to get a glimpse of what the iron import machinery may look at the cell surface, we used our crystals structures to model the TbpA-TbpB-hTF triple complex, showing TbpA and TbpB interacting with hTF simultaneously. This was further supported by an EM 3D reconstruction of purified triple complex. In conclusion, our structural and biochemical studies allow us to propose a mechanism by which Neisseria are able to methodically hijack and import iron from hTF for its pathogenesis.

NIEHS
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Protein Structure/Structural Biology

Structural Basis of DNA Ligase Proofreading by Aprataxin with insights into AOA1 Neurodegenerative Disease

Maintenance of genomic integrity is completed in the ultimate step of DNA replication and repair transactions when eukaryotic ATP-dependant DNA ligases seal DNA nicks in a three step reaction: 1) adenylation of a ligase active site lysine, 2) transadenylation of DNA 5'-phosphate, and 3) phosphodiester bond formation with AMP release. This biologically critical process can fail at the last step, leaving 5'-adenylated DNA termini when ligases engage nicks harboring DNA-distorting adducts and DNA repair metabolites including common products of abundant cellular oxidative DNA damage. DNA 5'-adenylation must be reversed to prevent persistence of DNA single strand breaks (SSBs) and genome instability. To protect genomic integrity, Aprataxin (APTX) repairs damaged 5'-adenylated DNA termini arising from failed DNA ligation events. The importance of APTX DNA processing functions in mammals is underscored by the fact that mutations in human Aprataxin (APTX) are linked to the autosomal recessive neurological disorder Ataxia with Oculomoter Apraxia 1. However, due to a lack of detailed structural information, the molecular basis for APTX catalytic reversal of 5’ adenylation damage, and how APTX is inactivated in the neurodegenerative disorders remain largely unknown. Here, we report X-ray crystal structure of a Schizosaccaromyces pombe APTX/dsDNA/Amp ternary complex that show how structural fusion of a HIT (histidine triad) nucleotide hydrolase fold with a Zif-268 related Zn-finger DNA binding scaffold assembles the APTX active site and dsDNA interaction surfaces. APTX-DNA complex structure illustrates how APTX senses DNA ends or nicks by capping the exposed base stack with a helical wedge. Employment of paired aromatic paddles and a conserved APTX "FPK-pivot" motif facilitates rotation of DNA 5'-adenylate orthogonal to the dsDNA duplex axis and into the active site pocket for de-adenylation repair. To dissect molecular determinants of APTX substrate recognition and catalysis we generated an extensive set of single amino acid substitutions based on APTX structures. Our results illustrate that obstruction of APTX DNA binding surface specifically impairs catalysis on adenylated DNA substrates, but not on nucleotide substrates such as Ap4A. Structures and mutagenesis support a HIT-Znf catalytic mechanism for AMP-adduct removal, and reveal mutations impacting protein folding, the active site pocket, and FPK-pivot underlie APTX dysfunction in neurodegenerative disease.

NIEHS

Wang, Huanchen

Postdoctoral Fellow

Protein Structure/Structural Biology

Substrate Specificity and Catalysis Mechanism of Inositol Pyrophosphate Kinase

The inositol pyrophosphates, IP7 and IP8, consist of phosphates and diphosphates closely packed around a six-carbon inositol ring. These molecules regulate a variety of cellular processes including apoptosis, vesicle trafficking, cytoskeletal dynamics, exocytosis, telomere maintenance, and adaptations to environmental stress. In this study we have asked how the reaction mechanism and stringent substrate specificity of the IP7 kinase (PPIP5K) has evolved despite the steric bulk and intense electronegativity of multiple phosphates in such a confined space. The answers to these questions come from our providing the first description of the crystal structure of an enzyme that synthesizes an inositol pyrophosphate. We have obtained 14 separate structures of PPIP5K to 1.7-2.0 A resolution in binary, ternary or quaternary complexes with either nucleotide, substrate, transition analog or product. Specificity of docking of either IP6 or 5-IP7 into the substrate binding pocket is ensured by an array of positively charged lysine and arginine residues. The importance of these key residues to substrate binding was verified by mutagenesis and by kinetic studies. ATP was deeply buried with only 9% solvent-accessible indicate that it is difficult to exchange with the bulk phase. The various structure complexes with the transition state analog, the reactant, and the product, provide possible snapshots of dynamic

reaction steps. After the IP7 substrate is bound, slight conformational changes compress the distance between the IP7 and ATP by about 1 A, and this facilitates nucleophilic attack to the gamma phosphate of ATP, and then the IP8 product leaves the active site. The magnesium atom in the transition state analog is 2.3A from the donor and 1.9A from the acceptor oxygen atoms and the angle is 166 degree among the three atoms, indicating a partial associative character of the transition state. This direct-in-line phosphoryl transfer mechanism rules out the possibility of a phosphoenzyme intermediate participating in the reaction. Lys-248 emerges as a key catalytically-essential residue and we find that it is not just spatially conserved across inositol kinases but also in cAMP dependent protein kinase A. Our studies not only provide us with an understanding on the catalytic mechanism but also they reveal a novel evolutionary link between inositol kinases and protein kinases in term of the phosphoryl transfer mechanism.

NCI-CCR
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Protein Structure/Structural Biology
Structural mechanism of CD4-independent HIV infection
CD4+ T-cells coordinate immune responses and their depletion is a hallmark of human immunodeficiency virus (HIV-1) and simian immunodeficiency virus (SIV) infection. Determining how HIV-1 enters target immune cells is paramount for development of an effective AIDS vaccine. HIV-1 and SIV enter T-cells by binding surface receptors, CD4 and chemokine co-receptors, triggering membrane fusion. Functional viral surface envelope glycoproteins (Env) trimers permit membrane fusion and subsequent entry. Env trimers are composed of non-covalently associating subunits of gp120, involved in binding cellular receptors, and transmembrane gp41, containing the fusion peptide. Using methods developed by our lab to classify and average noisy data obtained by cryo-electron tomography (cryo-ET), we found that the overall molecular architectures of both HIV-1 and SIV native Env trimers on intact viruses are structurally similar at resolutions of ~ 20 Å. When trimeric Env is bound to soluble CD4 and a coreceptor surrogate (17b), there is a dramatic conformational change that results in an opening of the gp120 propeller at the apex, which we believe results in exposure of buried regions of gp41. Upon HIV-1 disease progression, immune responses are repressed due to CD4+ T-cell depletion. With immune system repression, variant HIV viruses can emerge. One such virus has relaxed cell entry requirements no longer requiring CD4 for cell entry (CD4-independence). In the brain, non-CD4+ cells are infected and correlates with AIDS-induced encephalopathy and dementia. We have now demonstrated using cryo-ET that a CD4-independent SIV obtained by cell passage displays a similar open conformation previously observed upon emulating viral entry. Previously protected, conserved gp120 regions are now exposed in the open conformation, available as epitopes to elicit antibodies - a necessity for a successful AIDS vaccine. We are now actively exploring the possibility that a constitutively open state may be ubiquitous to other CD4-independent viruses isolated from productive viral infection. Preliminary investigations using cryo-ET are already underway to test this hypothesis using a number of matched pairs of CD4-dependent and CD4-independent SIV and HIV-1 variants. Administration of immunogens that mimic the open state of trimeric Env could potentially result in elicitation of novel broadly neutralizing antibodies reactive to both CD4-dependent and CD4-independent HIV strains.

NIMH
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Psychiatry

5-HTTLPR Serotonin Transporter Polymorphism Predicts Valence-dependent Risk Preference Shift and Related Amygdala Activity

The evaluation of risk is essential to human decision making. The potential for loss causes individuals to accept more risk to avoid an aversive outcome, a phenomenon known as the Reflection Effect. Given the link between risk perception and anxiety, determining the neural basis of the Reflection Effect has significant implications for understanding anxiety. We conducted a neuroimaging genetics study using fMRI and a decision-making task derived from behavioral economics in which gain and loss decisions are presented separately. During scanning, participants (N=69; 30 males and 39 females) were repeatedly presented with two options: a risky prospect in which subjects had a 50% chance of receiving one of two displayed values and a certain prospect in which subjects would receive the displayed amount. In males we show that allele load of the short (s) variant of the 5-HTTLPR serotonin transporter polymorphism predicts greater preference for risk in the face of potential losses relative to gains (Pearson's r = 0.48, p = 0.008). Furthermore, male ss carriers exhibit decreased amygdala activation to high risk potential loss while ll carriers exhibit increased activation (Pearson's r = -0.72, p = 8.4x10-6). One possible interpretation of these data is that s-allele load of 5-HTTLPR actually predisposes males to higher risk seeking when faced with monetary losses relative to their risk preference when faced with monetary gains by altering the amygdala's response to higher versus lower levels of risk. Traditionally ss carriers have been associated with high trait anxiety. Although the term "high anxiety" suggests ss carriers should be averse to risk, our results suggest that a certain loss is so aversive to an ss male that he would be willing to assume greater risk in order to avoid it. These data also illustrate how a behavioral trait (e.g., anxiety) and its underlying genetic correlate can be misunderstood if the context is not explicitly defined. This study adds to our understanding of the relationship of 5-HTTLPR and anxiety and is a key step in deciphering how the brain attempts to make optimal decisions in the face of uncertainty.

NIDA
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Psychiatry

What are the phenotypes of VTA neurons responsive to cocaine?

Given intravenously cocaine can either inhibit or excite VTA neurons. However, our knowledge of what types of VTA neurons are responsive to cocaine is based upon indirect electrophysiological evidence. For many years the VTA has been characterized as a structure with two types of neurons with distinguishable electrophisiological signatures: GABA and DA neurons. Recent anatomical findings indicate a third phenotype, glutamate neurons, is also present in the VTA. Moreover, recent electrophysiological studies have shown that some neurons that fulfill the electrophysiological criteria of a DA neuron are, in fact, non-DA. We set the task of phenotyping VTA neurons responsive to intravenous cocaine administration. We recorded VTA neurons in urethane-anesthetized rats. A glass electrode filled with neuronbiotin was used to juxtacellularly label neurons after we recorded changes in discharge rate resulting from intravenous cocaine injections. We labeled 33 VTA neurons: 11 DA neurons (immupositive for tyrosine hydroxylase) and 22 non-DA (immunonegative for tyrosine hydroxylase). We found no significances in action potential duration or discharge rate between DA and non-DA neurons. Of the DA neurons, 7 (63%) were inhibited while 4 (37%) were excited. Of the non-DA neurons, 8 (37%) were inhibited, 6 (27%) were excited, 2 (9%) were excited followed by inhibition, and 6 (27%) were non-responsive. This work provides the first evidence of the effects of intravenous cocaine on histochemically identified DA neurons; it is also the first evidence of the effects of intravenous cocaine on identified non-DA neurons in this region. The continuation of this project involves differentiation of the non-DA sub-populations by coupling juxtacellular labeling with in situ hybridization. We have successfully refined methods that are effective for identifying expression of mRNA for vGluT2 (marker

for glutamate neurons) and for GAD (marker for GABAergic neurons) and we are now collecting electrophysiological data from neurons that will be identified with these markers. Unpublished data from our group and recently published data from our group and others confirm that GABA and glutamate VTA neurons can each locally regulate DA neurons and that these GABA and glutamate neurons project in parallel with the mesocorticolimbic DA system. Taken together, these findings suggest rich DA, glutamate, and GABA interactions in a primary link in brain reward circuitry.

CC
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Other
Radiology/Imaging/PET and Neuroimaging
Automatic Detection and Precise Quantification of Infectious Lung Diseases from CT Scans
Respiratory tract infections are a leading cause of death worldwide (e.g., swine-origin H1N1, pneumonia, tuberculosis, etc.). Although radiology serves as a primary diagnostic method for assessing respiratory tract infections, visual analysis of CT scans is restricted by low and a limited capacity to assess severity and predict patient outcomes. In this study, we propose a novel computer assisted detection (CAD) system to reduce these limitations. In particular, our system is able to detect and quantify characteristic imaging pattern (Tree-in-Bud (TIB) opacity) often seen in pulmonary infections. The proposed method is not only the first CAD system in the literature automatically detecting TIB patterns from CT scans, but it also includes novel discriminative approaches to quantify infectious lung diseases precisely. The proposed CAD system includes the following steps. 1) Based on the texture and shape properties of the abnormality patterns in the images, a novel candidate pattern selection method is introduced to identify possible abnormal patterns. 2) A novel shape feature is presented to extract complex shape information from local regions enclosing candidate patterns. Unlike the popular feature extraction methods (which usually extract hundreds and even thousands features per region) in CAD systems, we extract only 8 features per region. 3) Extracted features are trained via support vector machines (SVM) classifiers such that discriminative properties of normal and abnormal imaging patterns are used to create a representative model for infectious diseases. 4) We detect abnormality patterns of any given image by testing their extracted features with the representative model created by SVM. We test our proposed system on 39 laboratory confirmed CTs of viral bronchiolitis human parainfluenza (HPIV) infection and 21 normal lung CTs. The proposed CAD system achieved a high detection rate with an overall accuracy of 90.96%. We compared our proposed feature extraction method with the state-of-the-art methods previously shown to be successful in CAD systems. We concluded that our proposed method is superior to those methods with statistically significant level of p=0.01. We also compared the computer score with the visual scores of two radiologists on a scale of 0-5. We observed through paired t-test that computer score (with our proposed CAD system) and visual score have significant correlations with a statistically significant level of p=0.01.

NIMH
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Novel imaging of nociceptin/orphanin FQ peptide receptors in brain and whole body of monkeys and humans using a new positron emission tomography radioligand, [11C]NOP-1A
Purpose: Nociceptin/orphanin FQ peptide (NOP) receptor is a new class of G-protein coupled opioid receptor linked to pathophysiology of many neuropsychiatric disorders such as drug abuse and anxiety. This receptor has not been imaged with PET. We developed a new PET radioligand, [11C]NOP-1A, that has high affinity (Ki = 0.15 nM) for NOP receptor and assessed its utility to quantify NOP receptors in

monkey and human brain, and estimated its radiation safety profile based on its biodistribution in humans. Methods: A pair of baseline and blocking PET scans was acquired on three rhesus monkeys and baseline brain PET scans were acquired on five healthy subjects for 2 hrs including arterial plasma measurements of concentration of parent radioligand 11C-NOP-1A. For blocking, a nonradioactive receptor antagonist (LSN2558114; 1 mg/kg i.v.) was administered prior to 11C-NOP-1A. One (1-T) and two-tissue (2-T) compartmental analyses were performed to determine distribution volume VT. Whole body PET scans were acquired for 2 hrs in nine healthy subjects. Regions of interest were drawn on ten source organs and radiation-absorbed doses were estimated by the Medical Internal Radiation Dose (MIRD) method. Results: After injection of [11C]NOP-1A, uptake of brain radioactivity in both monkey and human was high (~5-8 SUV-Standard Uptake Value) followed by quick washout. Brain time-activity curves for monkeys were well fit using 1-T whereas those for humans fitted better by 2-T. VT (mL/cm3) in monkeys was highest in neocortex (~20) and lowest in cerebellum (~13) compatible with known distribution of NOP receptor. LSN2558114 blocked ~50 – 70% of uptake and reduced VT in all brain regions to ~7 indicating that ~2/3 of activity was specific binding. VT in humans was highest in putamen (~11) and lowest in cerebellum (~6). In addition, VT was well identified within 60 min of injection and stable for the remaining 60 min indicating only parent radioligand entering the brain. The whole body scans showed radioactivity distributed in brain, peripheral organs expressing NOP such as heart, pancreas and spleen, and organs of metabolism and excretion such as liver and kidneys. Gall bladder had highest radiation exposure and the effective dose was 4.3 uSv/MBq. Conclusions: [11C]NOP-1A is a promising ligand to reliably quantify NOP receptors in brain. The radiation-absorbed dose in humans is low and similar to that of other 11C-labeled radioligands allowing multiple scans.

CC

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Mild hyperthermia using Magnetic Resonance-guided high intensity focused ultrasound (MR-HIFU) enhances drug delivery of low temperature sensitive liposome (LTSL) in a rabbit Vx2 tumor model

Introduction: MR-HIFU allows for noninvasive precise temperature control under image guidance. MR-HIFU has been traditionally used for ablations (T>60?C), but may be designed to deliver mild hyperthermia (40-45?C) for delivery of drugs using LTSL. The objectives of this study were 1) to develop a clinically relevant mild hyperthermia heating algorithm using MR-HIFU, and 2) to investigate the combination of MR-HIFU and LTSLs to enhance delivery of doxorubicin in a Vx2 rabbit tumor model. Methods: 15 New Zealand white rabbits, with Vx2 tumors (> 1 cm diameter) grown in the superficial thigh muscle, were randomly assigned into three treatment groups (dose = 5 mg/kg doxorubicin, IV): 1) free doxorubicin, 2) LTSL (ThermoDox®, Celsion Corp.,USA) and 3) LTSL+MR-HIFU. For the LTSL+ MR-HIFU group, rabbit tumors were heated with MR-HIFU (Sonalleve, Philips Healthcare,USA) to a target temperature of 40-41°C for a total of 30 min completed within 1 hour after LTSL infusion. Real-time feedback control of temperature in the target region was achieved using PRF-based temperature imaging and a binary control algorithm. Thermal data were analyzed for spatial offset (difference between targeted location and actual center) and temperature accuracy, as well as for homogeneity of heating. Four hours after treatment, tumors and organs were harvested. High pressure liquid chromatography (HPLC) analysis of the tumor and organ homogenates was performed to determine doxorubicin concentration. Treatment groups were compared for differences in mean doxorubicin concentration using ANOVA followed by Neuman-Keul's multiple comparison test. Results and Conclusion: Sonications of Vx2 tumors resulted in accurate temperature control (mean = 40.5°C). The heating was homogeneous in the target region (SD = 1°C) with a spatial offset of 1.2 mm. The tumor doxorubicin concentrations (mean ±SEM) were 2.96 ± 0.5, 7.48 ± 1.1, 32.4 ± 10.4 µg doxorubicin / g

tissue for free doxorubicin, LTSL and LTSL+MR-HIFU, respectively. The combination of LTSL and MR-HIFU mediated hyperthermia resulted in significantly higher tumor doxorubicin concentration compared to the other treatment groups (p<0.05). Combining MR-HIFU heating with LTSL yielded 4.3-fold greater doxorubicin concentration compared to treatment with LTSL alone. Biodistribution data in non-tumor organs/tissues were similar between treatment groups. This image-guided drug delivery technique has potential for clinical translation.

CC
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Computer-aided Teniae Coli Detection Using Height Maps from Computed Tomographic Colonography Images
Computed tomographic colonography (CTC) is an emerging minimally invasive technique for colonic polyps and cancer screening. Computer-aided diagnosis (CAD) system enables radiologist to identify colonic polyps more easily and accurately. Teniae coli are three bands of longitudinal smooth muscle on the colon surface. They are parallel, equally distributed on the colon wall, forming a triple helix structure from the appendix to the sigmoid colon. Teniae coli are important anatomical landmarks on human colon, which can serve as references for guiding virtual colon navigation and colonic polyp registration. We propose a novel method for teniae coli detection on CTC. Our method consists of four major steps. The first step is to obtain 2D height map from 3D colon. The 3D colon surface is unfolded and stretched to a rectangular region, then later on projected to 2D height map. The height map records the elevation of colon surface, where haustral folds correspond to high elevation points and teniae coli to low elevation points. In the second step, the height maps are sent into 2D Gabor filter banks, where images are characterized by their responses to a set of orientations and special-frequency selective Gabor filters. We used 3 scales and 4 orientations Gabor filter banks in our method. The maximum filtering responses from each scale and orientation are combined to form a feature image, which highlights the fold region versus the non-fold region. The third step is to identify fold centers from the feature image. We used local maxima and thresholding to locate the horizontal and vertical coordinates of the fold centers. The final step is to extract teniae coli, which are located where the haustral folds meet. The running path of folds can be generated by connecting fold centers. Teniae coli are extracted as the medial lines running between fold paths. We tested our algorithm on a CTC dataset with 7 cases. An experienced radiologist manually labeled the teniae coli as the reference standard, which is used to evaluate the performance. Root mean square error (RMSE) was employed for assessment. We normalized RMSE with the circumference of colon to obtain a percentage error rate. The proposed method achieved an average normalized RMSE of 5.66%, with a standard deviation of 4.79%. The average angle between detected teniae and reference standard is about 11 degrees. The error rate of our method is very low and looks promising for clinical evaluation of colon disease.

NINDS
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Characterization of chronic multiple sclerosis lesions at high field MRI
OBJECTIVE: The increased resolution and magnetic susceptibility contrast of high field MRI offer opportunities to study the pathological process underlying Multiple Sclerosis (MS). The pathological hallmark of MS is the classical demyelinated plaque. Preliminary high field data show a varying appearance of the prevalent white matter lesions, suggesting a diverse underlying histopathology. To

investigate the sources underlying this contrast variation, we compared in-vivo and post-mortem MRI with histological staining. METHODS: High resolution susceptibility-weighted 7T images were obtained from 21 MS patients. 2D multi-echo Gradient Echo acquisition was performed with 0.3×0.3 mm2 resolution and TE = 15, 30 and 45 ms. Quantitative R2* (=1/T2*) maps were obtained by using exponential fitting. Homodyne filtering method was used to obtain unwrapped phase images. Contrast patterns in phase and R2* images were investigated in 220 areas defined as chronic lesions from conventional T2-weighted Fluid Attenuated Inversion Recovery, T2-weighted and T1-weighted Spin Echo images. In addition, post-mortem MRI of MS tissues were performed at 7T and 11.7T and compared with immunohistochemical stains specific for myelin, iron, and ferritin. RESULTS: The majority (60.5%) of the identified lesions had a normal phase and reduced R2*. A substantial fraction of the lesions (38.1%) had negative phase shift (i.e., increased MRI resonance frequency) either uniformly or at their rim and a various appearance in R2*. These two lesion contrast patterns were reproduced in the post-mortem study. Comparison with histology showed that while R2* reduction corresponded to severe loss of both iron and myelin, negative phase shift corresponded to focal iron deposits with myelin loss. The peripheral phase rings are consistent with the histochemical stain data showing iron deposition at the periphery of a subset of lesions. DISCUSSION: Combined analysis of 7T R2* and phase data may help in characterizing the pathology of MS lesions. The observed R2* decreases suggest profound myelin loss, whereas negative phase shifts suggest a focal iron accumulation. Lesions areas with reduced R2* and normal phase may have substantial unrecoverable reduction in myelination and axonal loss. Negative phase shift and normal or increased R2*, often found at the rim and periphery of lesions, may indicate ongoing myelin repair processes that are supported by substantial iron stores in the form of ferritin.

NIMH
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Individual metabolite-corrected image-derived input function for neuroreceptor tracers
Positron emission tomography (PET) typical quantifies protein targets by measuring the amount of radioligand taken up by the organ divided by the amount of radioligand delivered(the input function). The input function is measured by arterial cannulation. Image-derived input function (IDIF) is an elegant method to avoid the arterial line and estimate the input function from serial images of the internal carotid artery. IDIF has two major challenges: 1) the concentration of radioactivity measured in the PET image of the carotid artery must be corrected for the blurring (partial volume and spill-in effects) of such a small structure and 2) separation of the parent radioactivity from that of radiometabolites. IDIF has been extensively used with [18F]-FDG, because it has no radiometabolites. However it has never been used in clinical protocols for neuroreceptor tracers, mainly because of the presence of radiometabolites which cannot be distinguished from the parent compound by the PET camera. We present a method to derive a metabolite-corrected IDIF for neuroreceptor tracers. Methods: The method was tested on 12 subjects who underwent a [11C](R)-rolipram PET scan. The carotid signal measured from the images was represented as a linear combination of the radioactivity from the blood and spill-in from the surrounding tissue [Ccarotid(t) = RC * Cblood(t) + SP * Csurround(t)]. Carotid and background regions of interest were manually drawn directly on the summed PET frames of the first two minutes and then copied to all the frames. Four blood samples were used to calculate the recovery (RC) and the spill-in (SP) coefficients by linear least-squares analysis. The parent concentration was obtained by fitting a monoexponential function through the parent/whole blood ratio calculated by HPLC analysis in those same four blood samples. Distribution volume (VT) was quantified using a Logan plot. VT obtained with standard arterial sampling was used as reference. Results: The IDIF method provided a VT estimate equivalent to that of arterial sampling (mean IDIF/sampling area under the curve ratio 1.03±0.04; mean

IDIF/sampling Logan-VT ratio: 1.00±0.06). Conclusion: We proposed a simple IDIF method to overcome in a single step the problems of partial volume, spill-in and metabolite correction for neuroreceptor PET studies. This method can substitute for multiple arterial blood sampling without reducing the overall accuracy of [11C](R)-rolipram quantification.

NCI-CCR
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Signal Transduction - General
Differential Effects of Dimerization on B-Raf and C-Raf Kinase Activity in Normal and Disease Signaling
Proper regulation of Raf kinase signaling is critical for normal cellular function as mutation of the Raf kinases can result in cancer and certain developmental disorders. Inhibitors to the high activity, oncogenic V600E-B-Raf are currently in clinical use; however, caution must be taken, given that the use of these inhibitors in cells lacking V600E-B-Raf can promote heterodimerization of B-Raf and C-Raf, resulting in pathway activation instead of inhibition. Thus, a full understanding of the Raf activation process is critical for the development of effective therapeutic stategies. Toward this end, we investigated the importance of dimerization in Raf activation and identified novel differences between B-Raf and C-Raf. In the context of normal cellular signaling, we find that growth factor stimulation induces B-Raf/C-Raf heterodimerization as well as B-Raf and C-Raf homodimerization, and increases the kinase activity of both B-Raf and C-Raf. To further explore these interactions, we utilized mutations in the dimer interface that either enhance (E586K in B-Raf and E478K in C-Raf) or prevent (R509H in B-Raf and R401H in C-Raf) Raf dimerization. Interestingly, these mutations had only a modest effect on the activity of B-Raf; however, the E-K mutation greatly enhanced C-Raf activity and the R-H mutation completely abolished C-Raf kinase activity. In addition, while expression of E478K-C-Raf had little effect on endogenous B-Raf activity, endogenous C-Raf activity was significantly elevated in cells expressing E586K-B-Raf. Knockdown of C-Raf was also found to have little effect on growth factor-mediated B-Raf activation, whereas knockdown of B-Raf dramatically inhibited C-Raf activation following stimulation, highlighting the dependence of C-Raf activation on B-Raf. Next, we examined the effects of dimerization on mutationally activated B-Raf and C-Raf proteins. Strikingly, although the dimer interface mutations affected the ability of all the mutant proteins to heterodimerize, they only altered the biological activity (as measured in focus forming assays) of B-Raf and C-Raf proteins with moderate to low kinase activity, but not B-Raf proteins possessing high kinase activity. Together, these data indicate that dimerization is important for Raf activation under normal signaling conditions as well as in the context of moderate to low activity disease-associated mutations and that blocking Raf dimerization may be a potential therapeutic treatment.

NCI-CCR
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Signal Transduction - General
Interaction and Competitive Binding between a Positive and Negative Regulator of RET Signaling
RET (Rearranged during Transfection) is a receptor tyrosine kinase (RTK) that is activated by glial cell-derived neurotrophic factors (GDNF) and is critical to neuronal and kidney development. RET mutations causing loss of function are associated with Hirschsprung's disease, while gain of function mutations are associated with thyroid carcinomas and mutiple endocrine neoplasias (MEN2A&B). Cbl proteins are a family of ubiquitin protein (E3) ligases that mediate RTK degradation and thereby serve as negative regulators of RTK signaling. Cbl-c (a.k.a. Cbl-3), the most recently identified of the human Cbl proteins, is restricted to epithelial cells and can ubiquitinate and downregulate RTKs, including activated RET and

EGFR. Using the multikinase small molecule inhibitor, Sorafenib, we show that Cbl-c constitutively binds RET, but requires receptor activation and Cbl-c phosphorylation for RET ubiquitination. Through yeast two-hybrid screening, we identified the LIM protein Enigma (a.k.a. PDLIM7) as a Cbl-c interacting protein. Enigma is an adapter protein for kinase anchoring to the cytoskeleton and has been shown to be required for the mitogenic signaling of the RET/PTC2 oncogene. The interaction between Cbl-c and Enigma was confirmed in mammalian cells through co-immunoprecipitation of both transfected and endogenous proteins. Interestingly, Enigma did not co-immunoprecipitate with the ubiquitous homologues c-Cbl or Cbl-b indicating that this interaction is specific to Cbl-c. Using an activated, transforming form of RET, RET-MEN2A, we show that Enigma prevents Cbl-c-mediated RET ubiquitination and downregulation. This inhibition of Cbl-c mediated ubiquitination by Enigma is specific to RET as Cbl-c-mediated EGFR ubiquitination was unaffected by the presence of Enigma. Enigma blocks recruitment of Cbl-c to activated RET and this blocking is mediated through RET binding as an Enigma mutant, lacking the RET binding site, does not block Cbl-c recruitment or subsequent RET ubiquitination. Our data suggest that Cbl-c mediates downregulation of activated RET receptor and that Enigma acts as a positive regulator of RET signaling by preventing Cbl-c recruitment and RET ubiquitination. This is further supported by confocal microscopy data which suggests that Enigma prevents the internalization of RET-MEN2A induced by Cbl-c. Together these data demonstrate a novel interaction between a positive and negative regulator of RET signaling.

NCI-CCR
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Signal Transduction - General
Discovery of a novel trimolecular complex that regulates Erk activation in T lymphocytes.
Bam32 is an adapter protein expressed in hematopoietic cells that has recently been shown to be a novel mediator of Erk signaling in T cells. To understand how Bam32 regulates Erk activation we investigated which proteins interact with Bam32. Upon CD3 stimulation, Bam32 interacted with the serine/threonine kinase Pak1, a well-know activator of Erk signaling. The Bam32-Pak1 interaction is mediated by an interaction with phospholipase C gamma 1 (PLC-g1). Furthermore, using knockdown approaches, we observed that Bam32-mediated Erk activation requires both Pak1 and PLC-g1 expression. The interactions between these proteins have been mapped. Binding of the PLC-g1 C-SH2 domain to Bam32 requires Serine 141 of the adapter, and the PLC-g1 SH3 binds one of the 5 proline-rich domains of Pak1. A phosphomimetic residue at position 141 of Bam32 increased formation of the trimolecular complex and augmented Erk activation. Overexpression of Bam32 enhanced the PLC-g1-Pak1 interaction and Pak1 phosphorylation on several residues. Thus we have uncovered a novel trimolecular complex that includes Bam32, PLC-g1 and Pak1 and works in a cooperative manner to activate Erk signaling. To further understand the nature of this complex, we investigated whether PLC-g1 lipase activity was required for Bam32-mediated Erk activation. Traditionally PLC-g1-mediated Erk activation in T cells is thought to be through the production of DAG and activation of RasGRP. In contrast, the lipase function of PLC-g1 was not required for the Bam32 pathway to activate Erk. PLC-g1 bound to Bam32 was not phosphorylated and Bam32 binding prevented normal PLC-g1 phosphorylation and diminished calcium flux. In fact, we found that Bam32 overexpression decreased the LAT-PLC-g1 interaction necessary for optimal PLC-g1 activation. Furthermore, Bam32 mediated Erk phosphorylation does not require LAT expression. The mechanism by which Pak1 is known to be activated is the disruption of inactive homodimers by the binding of small GTPases Cdc42 and Rac1. PLC-g1 overexpression disrupted Pak1 dimers and recombinanat PLC-g1 SH3-GST also dissociated Pak1 dimers in an in vitro assay, thus supporting a role for PLC-g1 in mediating Pak1 activation. In conclusion we have

an uncovered a novel LAT-independent Bam32-nucleated pathway that positively regulate Erk signaling in T lymphocytes.

NCI-CCR
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Signal Transduction - General
Ablation of Smurf2 reveals an inhibition in TGF-beta signaling through mono-ubiquitination of Smad3
TGF-beta signaling is regulated by post-translational modifications of Smad proteins to translate quantitative difference in ligand concentration into proportional transcriptional output. Thus, fine tuning of Smads activity is crucial for TGF-beta signal transduction. Smad ubiquitination regulatory factor 2 (Smurf2) is a HECT E3 ubiquitin ligase and previous studies in cell culture systems suggested that Smurf2 induces Smads' polyubiquitination and proteasomal degradation, but whether this mechanism operates under physiological conditions is not clear. Here, we generated mice harboring a target-disrupted Smurf2 allele. Using primary mouse embryonic fibroblasts (MEFs) and dermal fibroblasts, we show that TGF-beta-mediated, Smad-dependent transcriptional responses are elevated in Smurf2-/- cells by both luciferase reporter assays and real-time PCR analysis. Surprisingly, loss of Smurf2 does not affect Smad protein activity and stability. Instead of promoting poly-ubiqutination and degradation, we found that Smurf2 actually induces mono-ubiquitination of Smad3 in vivo. This mono-ubiquitination depends on Smurf2 E3 ligase activity and Smad3 PY motif. Further in vitro peptide binding assay and isothermal titration calorimetry analysis revealed that phosphorylation of T179, immediately upstream of the Smad3 PY motif, enhances Smurf2 and Smad3 interaction. Consistent with the requirement for Smurf2 binding, T179 phosphorylation also enhances Smurf2 induced Smad3 ubiquitination. By using a series of deletion mutants and making point mutation, we mapped Smad3 mono-ubiquitination sites to the lysine residues at the MH2 domain. To test whether the ubiquitination affects the ability of MH2 domain in mediating Smad complex formation, we examined the homo- or hetero-complex formation in the presence or absence of Smurf2. We found that Smurf2 inhibits Smad complex formation. Further GST pull-down assay directly confirmed that Smad3 mono-ubiquitination induced by Smurf2 blocks Smad interacting sites and destabilizes Smad complex. Therefore, our findings reveal a new mechanism through which Smurf2 negatively regulates TGF-beta signaling by attenuating the activity of Smad3 rather than promoting its degradation.

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Other
Signal Transduction - G-proteins and Ion Channels
Effects of Regional Deficiency of G Protein (Gsa) in the Hypothalamus on Energy Homeostasis
The ubiquitously expressed G protein stimulatory subunit Gsa couples hormones and receptors to generate cAMP. Heterozygous loss-of-function mutations of Gsa cause a monogenic obesity disorder, Albright hereditary osteodystrophy, but obesity only develops when the mutation is on the maternal allele. Likewise, mice with maternal germline Gsa mutations (but not paternal) develop obesity and diabetes. This is due to the fact that the Gsa gene GNAS undergoes imprinting with Gsa being expressed primarily from the maternal allele in specific tissues. The central melanocortin receptors (MC4R) couple with Gsa to reduce food intake and increase energy expenditure. We have shown in heterozygous brain-specific Gsa knockout mice that the parent-of-origin effects are due to Gsa being preferentially expressed on the maternal allele within the central nervous system (CNS) including the paraventricular nucleus of the hypothalamus (PVN). Mice with disruption of the maternal Gsa allele throughout the CNS develop severe obesity and diabetes resulting from reduced energy expenditure but no change in food

intake. In the present study, we further investigated effects of disrupted Gsa expression in specific nuclei expressing MC4R on energy balance by generating mice with Gsa deficiency in PVN (PVNGsKO) using Sim1-cre or in the ventromedial hypothalamus (VMHGsKO) using Sf1-cre. Maternal (but not paternal) deletion of Gsa in PVN (mPVNGsKO) led to modest obesity and insulin resistance with reduced metabolic rate without altering food intake, confirming that Gsa imprinting in PVN contributes to the metabolic phenotype. In contrast, VMHGsKO mice with Gsa deletion in either maternal or paternal, or both alleles showed no metabolic phenotype under regular chow diet and no molecular evidence for Gsa imprinting in VMH. After being fed with high fat diet (HFD), mPVNGsKO mice gained more weight than control littermates. Interestingly, mVMHGsKO mice gained significantly less weight with less fat accumulation than control mice, and had improved glucose tolerance and increased insulin sensitivity. Gene expression of UCP1 in brown adipose tissue and UCP3 in muscle tended to be lower in mPVNGsKO mice fed with HFD but was significantly increased in mVMHGsKO mice, suggesting an enhanced adaptive thermogenesis in mVMHGsKO mice. These data indicate that Gsa signaling in different nuclei plays divergent roles in regulation of energy expenditure in response to excess nutritional challenge.

NIAID
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Signal Transduction - G-proteins and Ion Channels
RGS16 attenuates T lymphocyte-mediated inflammatory responses to helminth pathogens
Chemokine receptors are G-protein coupled receptors (GPCRs) that direct immune responses and inflammation. Regulators of G-protein Signaling (RGS) proteins negatively regulate GPCRs by enhancing GTPase activity of Ga, catalyzing its deactivation. RGS16, an inhibitor of chemokine responses expressed in activated T lymphocytes, inhibited allergic pulmonary inflammation in transgenic mice by reducing lymphocyte migration to the lung in response to ovalbumin sensitization and challenge. Lymph node (LN) T cells polarized to T helper type 2 (TH2) phenotype in vitro expressed more RGS16 than naïve cells. RGS16-deficient TH2 cells migrated more to the TH2-associated cytokine CCL17 than did wild-type cells, suggesting that RGS16 is a physiologically relevant regulator of TH2 trafficking. We investigated how the loss of RGS16 affected TH2 cell migration and cytokine production to helminth antigens in vivo using Rgs16 knockout (KO) mice. Mice were sensitized to Schistosome egg antigen (SEA) i.p. and challenged with S. mansoni eggs i.v. 14 days later, which produces a synchronized granulomatous fibrotic reaction in the lung. Lungs of Rgs16KO mice had more fibrosis (collagen deposition) and eosinophil infiltration than those of C57BL6WT mice, and developed larger granulomas. LN cells from RGS16KO mice produced more TH2 (IL-4, -5,-10,-13) and TH17 (IL-17) cytokines than those from WT mice upon antigen restimulation. We detected more Il17 expression and reduced expression of a suppressor of cytokine signaling (Socs2) in the lungs or spleen, respectively, of RGS16KO mice compared to WT, suggesting that dysregulated cytokine signaling in the absence of RGS16 could account for enhanced production of cytokines leading to fibrosis. Although overall numbers of CD3+CD4+ and B220+ cells were similar in challenged WT and RGS16KO lungs, tissues from RGS16KO mice had more CD4+IL-13+ cells relative to IFNg-producing CD4+ cells, representing increased TH2 skewing in the absence of RGS16. Moreover, in contrast to WT lungs, where CD3+ or B220+ cells were detected primarily within granulomas, T and B cells in KO lungs were localized predominantly at the periphery of granulomas, as well as in dense perivascular/peribronchial aggregates. Collectively, these results suggest that RGS16 controls both effector lymphocyte cytokine production and localization in complex ways after challenge with a helminth antigen, leading to attenuated TH2 and possibly TH17 immune responses.

NIDCR
Lombaert, Isabelle

Visiting Fellow
Stem Cells - General
The expansion of progenitor cells during organogenesis requires c-Kit/Fgfr2b-dependent transcription factor expression
Radiotherapy is an effective treatment for head and neck cancers. However, co-irradiation of the salivary glands often occurs whereby progenitor cells no longer replace secretory acinar cells, leading to a permanent loss of saliva production. Recently, serial transplantation of c-Kit+ submandibular gland (SMG) stem/progenitor cells regenerated irradiated SMGs in mice. However, the function of c-Kit signaling during regeneration is not understood. We hypothesize that c-Kit signaling is required for the maintenance and expansion of progenitor cells in the SMG. During early SMG development, epithelial end buds express c-Kit whereas its ligand, stem cell factor (SCF), is produced by the surrounding mesenchyme and end buds. Importantly, in the absence of Fgf10 or Fgfr2b the SMG does not develop. Here, we demonstrate that Fgf10/Fgfr2b signaling is essential for the survival and proliferation of c-Kit+ end bud progenitors by rapidly regulating SCF and c-Kit. Knockdown of c-Kit expression using siRNA significantly downregulated transcription factors (TFs) known to be downstream of Fgf10/Fgfr2b-MAPK signaling in the SMG: Etv4, Etv5, c-Myc and Sox10, which are involved in progenitor cell maintenance and self-renewal in other systems. Exogenous SCF did not regulate these TFs, however, stimulation of Fgf10-treated epithelium with SCF resulted in a rapid increase in gene expression within 3hrs. This increase was mediated via a c-Kit-specific PI3K/MAPK pathway that is dependent on Fgfr2b signaling. Interestingly, addition of SCF to Fgf10-cultured epithelia for 24hrs also increased the number of Keratin14 (K14)-expressing cells, which are a SMG progenitor population that co-expresses TFs such as Sox10. We used FACS analysis to demonstrate that treatment of intact SMGs with SCF expanded the c-Kit, K14 and Sox10-expressing cells compared to the control. In summary, Fgf10/Fgr2b signaling maintains c-Kit progenitor cells during development by regulating SCF and c-Kit expression. Subsequently, SCF/c-Kit expands K14+ end bud progenitors by rapidly enhancing gene expression of TFs cMyc, Sox10, Etv4 and Etv5. These data may have implications in other Fgfr2b dependent branching organs. Our data highlights the necessity of SCF/c-Kit signaling to control the appropriate number of progenitor cells, which will be important for both development and during organ regeneration after injury.

NIAID
Sweeney, Colin
Postdoctoral Fellow
Stem Cells - General
Zinc Finger Nuclease Mediated Safe Harbor Targeted Gene Transfer in Patient iPSCs Functionally Corrects X-linked Chronic Granulomatous Disease
X-linked chronic granulomatous disease (X-CGD) is a defect of neutrophil microbicidal reactive oxygen species (ROS) production resulting from gp91phox deficiency, caused by a variety of mutations throughout the CYBB gene. We have developed induced pluripotent stem cells (iPSCs) from patients with X-CGD, and have demonstrated that mature neutrophils differentiated from X-CGD iPSCs lack ROS production, reproducing the defining characteristic phenotype of this disease. Gene therapy of X-CGD using retroviral or lentiviral vectors carries a risk of mutagenesis due to vector insertion near oncogenes. Homologous recombination can be used for safe gene delivery to a specific site in the genome, but has very low efficiency. Utilizing zinc finger nucleases to facilitate homologous recombination, we have achieved high-efficiency targeted transfer of a therapeutic CYBB minigene into the safe harbor AAVS1 locus in X-CGD iPSCs from two patients with CYBB mutations in either exon 5 or in the splice donor site of intron 10. Following puromycin selection for targeted gene transfer, iPSC clones were screened for evidence of off-target insertions or other changes. We observed that 78% (28/36) of corrected clones

contained vector insertion into a single AAVS1 allele without off-target inserts. Of these, approximately 50% contained small deletions or insertions at the non-targeted wild-type AAVS1 allele due to non-homologous end joining, while no mutations were detected at the top 10 predicted off-target sites for the AAVS1 zinc finger nucleases. After screening for single copy targeted insertion without additional genetic changes, corrected clones were found to exhibit sustained expression of gp91phox and maintained normal karyotype and full pluripotency as measured by pluripotency markers and in vitro embryoid body and in vivo teratoma assays. Upon neutrophil differentiation, the corrected clones exhibited functional restoration of ROS production (27-42% of differentiated cells positive for oxidase by dihydrorhodamine flow cytometry assay, compared to 0.4-0.9% for neutrophils from uncorrected X-CGD iPSCs and 23-29% for neutrophils from normal control iPSCs), at fluorescence intensity levels up to 80% of normal controls. Our findings demonstrate that precise safe harbor minigene targeting can be used for the correction of X-CGD using zinc finger nucleases in iPSCs from patients with different CYBB mutations, an approach that might also be applied to other monogenic diseases.

NCI-CCR
Xiao, Ying
Postdoctoral Fellow
Stem Cells - General
Hedgehog signaling creates a perineural stem cell niche essential for maintaining sensory touch domes in skin
The skin is an essential barrier and is also a sensory organ. In mammalian skin the interfollicular epidermis (IFE) contains specialized epithelial structures termed touch domes (TD) that are composed of keratinocytes in juxtaposition with neuroendocrine Merkel cells (MC). MCs are involved in slow adapting touch sensation. Located within the TD basal layer, MCs form close associations with myelinated sensory nerve endings. There is ongoing cellular turnover in TDs, however, the nature of the stem/progenitor population that maintains the TD and its MCs remains unclear. In many adult tissues, Hedgehog (Hh) signaling regulates quiescent stem cell populations. Using reporter mice with LacZ knocked into the locus coding for Gli1, a Hh target gene, we determined that the basal cell layer of the specialized TD epithelium is composed of Hh-responding cells. Similarly, LacZ reporter mice were used to show that the TD also expresses Gli2 and Gli3, transcription factors that are obligate effectors of Hh signaling. This is in contrast to the remainder of the IFE that was negative for Gli1, Gli2 and Gli3, indicating an absence of Hh signaling. Thus it is likely that Hh signaling regulates TD biology. Next we used Gli1CreER/+ ; R26RLacZ/LacZ mice to fate map the Gli1-expressing cells in TDs. Labeled cells from the TD basal layer contributed to the overlying epithelium and to MCs indicating that Hh-responding progenitors provide both the epidermis and sensory cells of the TDs. When these mice were aged, the labeled cells were retained in the TDs for up to 1 year indicating that self-renewing Gli1-expressing stem cells maintain the TDs. Importantly, labeled cells in the TDs did not migrate out into the surrounding IFE, demonstrating that the TDs is a unique lineage in the skin that is maintained by its own stem cell population. Finally, we have shown that Sonic Hedgehog (Shh) expressing sensory nerves were the source of Hh signaling to TDs, as cutaneous denervation eliminated Gli1 expression from TDs, followed by the loss of TDs themselves. Therefore the TD with its MCs is a unique epithelial lineage within the skin that is maintained by Gli1-expressing stem cells that are dependent on a perineural stem cell niche where nerves provide Shh signaling. These results suggest that neurons trophically maintain the specialized sensory epithelia they are associated with by signaling directly to lineage specific epithelial stem cells.

NCI-CCR
Ji, Junfang
Visiting Fellow

Stem Cells and Cancer

Identification of MicroRNAs Specific to Hepatic Cancer Stem Cells but Not to Normal Stem Cells by Small RNA Deep Sequencing

MicroRNAs (miRNAs), small noncoding RNAs that are post-transcriptional gene regulators, are functionally linked to normal stem cells, cancer stem cells (CSCs) and cancer therapy. CSCs are cancer cells that possess characteristics shared with normal stem cells and can give rise to a new tumor in xenotransplant assays. Although drugs targeting CSCs hold promise in eliminating cancer burden, normal stem cells are likely to be targeted due to their similarities with CSCs. Hepatocellular carcinoma (HCC) is an aggressive and heterogeneous human malignancy attributed to the presence of hepatic cancer stem cells (HepCSCs). Isolated EpCAM+ HCC cells, using an EpCAM-specific antibody, are HepCSCs with properties of normal hepatic stem cells. We hypothesized that certain miRNAs are exclusively altered in EpCAM+ HepCSCs and are ideal molecular targets for developing effective HCC therapies. We used next generation sequencing technology to profile miRNAs, since it provides deep coverage and base level resolution to quantify miRNAs and to identify novel miRNAs. We made 16 small RNA libraries with SOLiD Total RNA-Seq and RNA Barcoding Kits. These libraries were derived from: a) EpCAM+ HepCSCs and corresponding differentiated EpCAM- HCC cells isolated from two human primary HCC specimens and two HCC cell lines; b) normal hepatic stem cells (EpCAM+; pluripotent stem cells) and their lineage restricted hepatoblasts (EpCAM+; bipotent progenitor cells) from two human fetal livers; c) primary hepatocytes (EpCAM-) from three liver donors; d) pooled embryonic stem cells from H1 and H9 stem cell lines. In our analysis pipeline, 35 nucleotide-filtered reads were aligned to the reference human miRBase, then to the human genome. The alignment yielded an average of 5.56 million high-quality uniquely mapped reads, of which 78.9% were aligned to miRBase. On average, we identified 98.0% of the known miRNAs (n=1049) within miRBase. The miRNA profiles were then compared between EpCAM+ and EpCAM- cells to identify unique miRNAs specific to HepCSCs. The results show that 28 miRNAs were specifically altered in EpCAM+ HepCSCs. We are currently examining miRNA mutations associated with HepCSCs. The functions of these miRNAs in HepCSCs will be further explored. Here we provided the most extensive profiling of miRNAs in HepCSC and identification of unique miRNAs in EpCAM+ HepCSCs might assist in developing treatments to specifically eliminate HepCSCs without sacrificing normal stem cells.

NCI-CCR

Santos, Margarida

Visiting Fellow

Stem Cells and Cancer

Chromatin modifications and hematopoietic stem cell regulation: implications for leukemic transformation and aging

Hematopoietic stem cells (HSCs) are unique cells with the amazing capacity to replace millions of blood cells that die every second throughout our lives. The balance between self renewal and differentiation of HSCs needs to be tightly regulated. Dysregulation of this process is the basis of cell transformation and leukemia, as well as aging of the hematopoietic system. It has been shown that the expression or silencing of specific genes controls this differentiation/self renewal balance and many of these genes have been found associated with transformation in humans and murine models. Chromatin modifications that affect gene expression were shown to be very dynamic during HSC differentiation but what specific factors regulate these epigenetic modifications is a question that remains largely unknown. This study is focused on PTIP (Pax- transactivation interacting protein), an epigenetic regulator component of the MLL3 (mixed-lineage leukemia 3)/MLL4 complex. To evaluate whether the PTIP/MLL3/MLL4 complex plays a role in HSC maintenance and differentiation, we are using mice with a conditional deletion of PTIP or MLL4 in HSCs. We observed that in the absence of PTIP, the HSC

population is no longer found in the bone marrow and there is extramedulary hematopoiesis. In the absence of MLL4, HSCs are present but in both PTIP and MLL4-deficient mice there is a skewing towards myeloid differentiation and signs of a myeloproliferative disease. Moreover, absence of PTIP or MLL4 causes elevated levels of reactive oxygen species in the HSCs, persistent DNA damage, cell-cycle dysregulation and dramatic defects in lymphoid precursor cells. Notably, these defects have also been observed in aged HSCs from mice and humans. Gene expression profiles showed a decreased expression of PTIP and other components of the MLL3/4 complex with age. We propose that HSC decline with age is a result of epigenetic dysregulation in which loss of PTIP and the MLL3/4 complex could play a crucial role. To explore this idea, we are characterizing gene expression in HSCs isolated from PTIP and MLL4 deficient mice and comparing these with changes in HSCs derived from old mice. We are also currently testing whether mice deficient for PTIP or MLL4 in the hematopoietic cells develop leukemia, in association with other mutations (ex. deletion of p53). In summary, our work provides insight into the factors that control HSC differentiation and dysregulation during leukemic transformation and aging.

NIA
Martin Montalvo Sanchez, Alejandro
Visiting Fellow
Stress, Aging, and Oxidative Stress/Free Radical Research
NQO1 Overexpression Increases Antioxidant Protection And Glucose Homeostasis In Mice
One of the most important benefits of Calorie Restriction (CR) is an improvement of the plasma membrane redox system. CR enhances the activities and content of antioxidant compounds known to decline with age. Previous studies from our laboratory have shown that the levels of expression and activity of NADH coenzyme Q oxidoreductase 1 (NQO1) are increased in rodent plasma membranes under long-term CR. This overexpression promotes quenching of free radicals and other oxidizing compounds, and it contributes to the regulation of cellular redox homeostasis, particularly the NAD/NADH ratio. We present here the characterization of NQO1 overexpression in transgenic mice produced in our laboratory. These mice harbor the rat orthologue of NQO1 which was cloned into a pRC/CMV-rDTD vector. The construction was stably incorporated into the genome and the gene expression was controlled by a promoter associated with human cytomegalovirus and the appropriate sequences for polyadenylation. NQO1 overexpression resulted in lower body weight gain under high fat feeding conditions (HF) and resulted in a myriad of beneficial physiological effects. Particularly interesting was an increase in oxygen consumption, which might explain the decreased weight gain under HF. In addition, overexpression of NQO1 prevented the onset of insulin insensitivity and it protected against protein oxidative damage. Furthermore, NQO1 KI mice showed an increase in the NAD/NADH ratio as well as ATP levels, but these increases were not associated with increased mitochondrial content. These metabolic changes in NQO1 KI mice lead to a decrease of inflammatory markers, improvements in insulin sensitivity decreased protein oxidation damage in HF and increased oxidative stress tolerance in an acute oxidative treatment of the superoxide producer diquat in a standard diet. These findings support that NQO1 overexpression, as seen in CR, has long-term benefits and prevents the deleterious effects of HF in mice. Our future research will determine the particular pathways and proteins that are involved in the induced benefits of NQO1 overexpression, the mechanisms underlying glucose homeostasis improvements and the long-term effects of NQO1 overexpression during an ongoing longevity study. We predict that NQO1 overexpression will lead to healthspan and lifespan benefits.

NIA
Vallabhaneni, Haritha
Visiting Fellow

Stress, Aging, and Oxidative Stress/Free Radical Research
Characterization of telomere phenotype in Nth1 deficient mice
Telomeres are the chromosome end structures composed of TTAGGG repeats that protect the chromosomes from the DNA damage response machinery. Oxidative stress induces oxidative base lesions, which accumulates during the lifespan of an organism and are thought to contribute significantly to aging. It has been proposed that oxidative base damage occurs at a higher rate in telomeres which then leads to telomere attrition and aging. However, the exact mechanism whereby damage leads to telomere attrition is not known. This study seeks to directly test whether elevated DNA damage leads to telomere length alterations. Oxidative base lesions are repaired primarily by a base excision repair pathway initiated by DNA glycosylases. OGG1 and NTH1 glycosylases remove oxidized purines or pyrimidines in dsDNA, respectively. Ogg1 and Nth1 deficient mice are excellent model systems to study telomere length regulation due to the accumulation of oxidative purines or pyrimidines. Previously, we have shown that murine cells deficient in the OGG1 glycosylase display multiple telomere defects. In the present study, we extended our analysis to include the effect of Nth1 deficiency on telomere maintenance. We used telomere-fluorescence in situ hybridization to analyze the length of the telomeres. We found that freshly isolated Nth1 deficient bone marrow cells and spleenocytes and cultured bone marrow cells showed decreased telomere length. Since Nth1 deficiency could lead to the accumulation of oxidized lesions, e.g. thymine glycols, that cause replication forks to stall, we examined fragile telomeres and telomere recombination processes that are proposed to form due to impaired telomere replication. Nth1 deficient cells showed an increased percent of fragile telomeres and T-SCEs (telomere-sister chromatid exchange) suggesting that oxidized bases in telomeric DNA do pose a problem to the replication of telomeres. Additionally, Nth1 deficient cells also showed increased double strand breaks and increased telomere dysfunction-induced foci as evidenced by increased 53BP1 foci, a marker for double strand breaks. Our studies indicate that oxidized pyrimidines may affect telomere replication and thus telomeric length homeostasis. Our studies pave a way to understand how oxidative DNA damage impacts telomere length, disregulation of which has been linked to human aging, premature aging syndromes, and cancer predisposition.

NHLBI
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Doctoral Candidate
Stress, Aging, and Oxidative Stress/Free Radical Research
Mitochondrial Acetyltransferase 1 (MAT1) and Sirt3 function as a 'nutrient sensors' regulating mitophagy
Mitophagy is a selective form of autophagy that recycles defective mitochondria. Defective mitophagy plays a role in cancer, diabetes and neurodegenerative disorders. Fasting induces mitophagy and may slow the onset of age related diseases. Fasting also activates the sirtuin deacetylases (Sirts). Mitochondrial function is modulated by acetylation status, and the only known regulators of mitochondrial acetylation are Sirt3 and Sirt5. We recently described the first mitochondrial acetyltransferase (MAT1) which counters Sirt3 mediated effects on respiration and ATP generation by acetylating complexes I and V. Since mitophagy is induced by fasting and the benefits of fasting are dependent on sirtuins, we hypothesized that Sirt3 augments and MAT1 attenuates mitophagy in a nutrient dependent manner. We first investigated the role of nutrient status on mitophagy. Liver mitochondria from fasted wildtype mice have augmented mitophagy, increased Sirt3 and lower MAT1 levels. However, fasted Sirt3 knockout mice fail to induce mitophagy. Mitochondria from HepG2 cells depleted of MAT1 show increased levels of the mitophagy markers LC3-II, p62 and ubiquitination, while Sirt3 siRNA attenuates levels. Confocal microscopy confirms increased co-localization of these markers to mitochondria with MAT1 knockdown. MAT1 depletion increases autophagosomes and autolysosomes

containing mitochondria as shown by both confocal and electron microscopy. Global autophagy is not increased with MAT1 knockdown, as measured by P-P70S6K and cellular LC3-II levels, suggesting selective mitophagy is induced by MAT1 siRNA. Dual depletion of MAT1/p62 or MAT1/parkin, an ubiquitin ligase, limits mitochondrial LC3-II levels, showing that p62 and parkin are necessary for MAT1 siRNA induced mitophagy. Mitochondrial ROS is linked to various disease states, and disrupted mitophagy is shown to increase ROS levels. We show that MAT1 knockdown limits ROS induction following rotenone treatment compared to control or Sirt3 depletion, suggesting that the augmented mitophagy by MAT1 depletion is protective against oxidative stress. Overall, fasting induces mitophagy, and we propose that MAT1 knockdown mimics fasting leading to augmented mitophagy by increasing Sirt3 activity. Functionally augmented mitophagy protects against oxidative stress possibly through enhanced mitochondrial turnover. MAT1 and Sirt3 may offer unique targets for disorders linked to defective mitochondrial homeostasis.

NCI-CCR
Arnaldez, Fernanda
Clinical Fellow
Tumor Biology and Metastasis
In vivo and in vitro alveolar rhabdomyosarcoma cell growth is affected by TNK2 expression
Rhabdomyosarcoma is the most common pediatric soft tissue tumor. Embryonal rhabdomyosarcoma (ERMS) is characterized by 11p15 LOH, and alveolar rhabdomyosarcoma (ARMS) exhibits the PAX3 (or PAX7)-FOXO1 translocation. Despite utilization of aggressive therapy, patients with recurrent or metastatic disease remain incurable. New therapies are needed to improve outcomes in this disease. To detect genes that are critical for rhabdomyosarcoma cell growth and survival, we performed a loss-of-function shRNA screen in ARMS and ERMS cell lines using a regulated shRNA library that was delivered to RMS cells using retrovirus. We hypothesized that genes whose loss of function significantly influence the growth and survival of ARMS cells with relatively no impact on ERMS cells represent a critical vulnerability or “Achilles' heel” for ARMS. We sorted our gene list using this approach. In this study, we identified TNK2 (tyrosine kinase, non-receptor 2; also known as ACK1) as a critical gene is ARMS cell survival and as a potential therapeutic target. TNK2 is a non-receptor tyrosine kinase that integrates signals from different membrane receptors, including integrins, EGFR and IGFR. Reported downstream targets include CDC42 and AKT. The TNK2 gene is located at 3q29. TNK2 amplification has been described in prostate, lung and breast cancer and its role in invasion and metastasis has recently been suggested. However, its role in rhabdomyosarcoma has not been previously explored. We confirmed that TNK2 is uniformly expressed RMS. Analysis of TNK2 expression in primary tumor samples of RMS using a publicly available database indicated that rhabdomyosarcomas with higher TNK2 expression show lower overall survival. We used a tetracycline-inducible shRNA construct to knock down TNK2 expression in rhabdomyosarcoma cells. TNK2 knock down resulted in significant decrease in cell growth in ARMS. We characterized the specificity of our findings using 5 different shRNA sequences targeting this gene delivered via lentivirus. In addition, we have been able to rescue the effect of an shRNA targeting the 3’UTR of this gene using TNK2 cDNA. We validated our in vitro data in an in vivo model using tumor xenografts. Downstream pathways under investigation include AKT, MAPK and CDC42. These data suggest that TNK2 could represent a therapeutic target in ARMS and highlight the role of loss of function shRNA screens to improve the understanding of tumor biology.

NCI-CCR
Goldberger, Natalie
Postdoctoral Fellow
Tumor Biology and Metastasis

Identification of miR-290-3p and miR-290-5p as tumor and metastasis suppressors in breast cancer
Several microRNAs (miRNAs) have been classified as regulators of breast cancer metastasis, yet few studies have examined how germline genetic variations may dysregulate miRNAs in turn creating a predisposition towards cancer and metastasis. To explore this concept, highly metastatic MMTV-PyMT mice were crossed with 25 AKXD (AKR/J x DBA/2J) recombinant inbred strains to produce F1 progeny displaying primary mammary tumors with varying degrees of pulmonary metastasis. The miRNA expression levels within all mammary tumors were evaluated by miRNA microarray and correlated with the metastatic index for each strain. Microarray analysis produced miR-290 as one of the top candidates. Further, when miR-290, containing both miR-290-3p and miR-290-5p, was ectopically expressed in the murine metastatic breast cancer cell line Mvt-1, a 70% reduction in mammary tumor burden and a complete suppression of lung metastasis was observed after orthotopic injection in FVB/N mice. Computational analysis identified Arid4b and Sox2 as top targets of miR-290-3p and miR-290-5p, respectively; both genes have been previously linked to breast carcinogenesis and metastatic potential. Next, the individual phenotypic effects of miR-290-3p and miR-290-5p were explored by generating Mvt-1 clones that either upregulated miR-290-3p or miR-290-5p. Confirmation that Arid4b is directly targeted by miR-290-3p was provided by luciferase reporter assay and by observing a 40% reduction in Arid4b expression in only the miR-290-3p expressing clone. In vitro models showed miR-290-3p increases migration and reduces proliferation, while miR-290-5p suppresses migration and has no affect on proliferation. When the clones were analyzed in vivo, miR-290-5p caused a 90% reduction in the number of lung metastases and a 60% reduction in mammary tumor burden, while miR-290-3p caused a 25% reduction in the number of lung metastases and a 90% reduction in mammary tumor burden. Overall, the unique in vitro and in vivo properties observed for each clone suggests miR-290-3p and miR-290-5p decrease breast cancer tumorigenesis and metastasis by targeting separate pathways, which may partially overlap. In conclusion, these results suggest germline genetic changes may exist that reduce miR-290 expression to create a predisposition towards breast cancer development and metastasis through the combined downregulation of both miR-290-3p and miR-290-5p.

NCI-CCR
Kalra, Neetu
Visiting Fellow
Tumor Biology and Metastasis
Anti-tumor efficacy of IMC-A12, a fully human anti-insulin like growth factor 1 receptor (IGF-1R) antibody, against malignant mesothelioma (MM)
MM is an aggressive cancer with limited treatment options and poor prognosis. Previous studies have shown that the interaction between IGF-1R and its ligands IGF-1 and IGF-2 play an important role in MM tumorogenesis. To exploit IGF-1R as a target for mesothelioma therapy we investigated the activity of IMC-A12 a fully humanized antibody against IGF-IR in 8 early passage mesothelioma tumor cells, 9 established MM cell lines and in vivo human mesothelioma tumor xenograft model. Quantitative real time PCR assay for genes involved in the IGF-axis showed heterogeneous levels when compared to the expression of genes in non-malignant mesothelioma cell line thus validated the importance of this pathway in MM progression. All early passage as well as established MM cell lines showed IGF-1R expression at the mRNA and protein levels. Using a quantitative electrochemiluminescence immunoassay we determined that there is considerable variability in IGF-1R expression ranging from 1-14 ng/mg of cell lysate. The number of IGF-1R cell surface receptors determined using Quantibrite bead assay showed ˜ 2,000-50,000 sites/cell. MM cells expressing >10,000 sites/cell had greater than 10% (range, 10% -40%) growth inhibition when treated with IMC-A12 (100 ug/mL). IMC-A12 treatment induced G0-G1 cell cycle arrest and inhibited the signaling through downstream pathways (Akt and Erk ½) of IGF-IR in the cell lines expressing high amount of IGF-IR but not in the cell lines, which had minimal

amount of IGF-IR expression. IMC-A12 treatment also induced ADCC (> 20% specific lysis) in those cell lines with IGF-1R expression of > 20,000 sites/cell. For in vivo experiment, the MM cell line, H226, stably transfected with the luciferase gene, was inoculated i.p into athymic nude mice. IMC-A12 (50mg/kg b.wt. i.p. twice a week for 60 days) treatment delayed tumor growth in athymic nude mice when compared to the control group (p< 0.05). Our results show that both early passage mesothelioma cells as well as established MM cell lines express IGF-1R but that there is a wide variability in the degree of IGF-1R expression. IMC-A12 exhibits anti-proliferative activity against these cell lines as well as induces ADCC, both of which are strongly co-related with IGF-1R cell surface expression. Single agent IMC-A12 also has significant in vivo anti-tumor activity. These results suggest IMC-A12 may be useful for treatment of MM and a phase II clinical trial of IMC-A12 is currently ongoing at the NIH.

NCI-CCR
Song, Yurong
Research Fellow
Tumor Biology and Metastasis
Inducible Astrocytomas in Genetically Engineered Mice: Delineation of Grade-specific Molecular Drivers in Tractable Preclinical Models
High-grade astrocytomas [anaplastic astrocytoma (AA) and glioblastoma (GBM)] remain fatal with no effective treatment. Understanding of major regulatory pathways involved in disease etiology will be critical for development of effective diagnoses and therapy. Genetically engineered mice (GEM) have been used effectively to probe gene function in astrocytoma. However, few studies have successfully queried cause and effect relationships solely in adult brain. Here we aimed to develop inducible models of low and high grade astrocytomas in GEM based on common specific pathways deregulated with high frequency in human GBM: disruption of pRb function (via dominant-negative inactivation), K-RasG12D activation (activated in human GBM by receptor tyrosine kinase or Raf activation or NF1 inactivation) and Pten inactivation. Lesion induction in these models was elicited by activation of CreERT2 driven by human GFAP promoter, after intraperitoneal 4OH-tamoxifen injection at 3 months of age. pRb inactivation alone was sufficient to initiate tumorigenesis (grade II disease), which was sufficient for maximum MAPK activation via endogenous K-Ras. Further activation of K-RasG12D predisposed to grade III astrocytic tumors (AA), which triggered PI3K downstream pathways and led to a selective pressure for Pten loss and disease progression. With high penetrance and reproducible timing, the combination of all three events predisposed to tumors with the common histological features of human GBM, including brain invasion, high mitotic indexes, and necrosis. This result demonstrates the importance of adult-specific induction of genetic lesions, since both K-Ras activation and Pten deletion have been shown to induce pathology when activated with constitutive GFAP-Cre expression. Sequencing analyses showed that a high fraction of the GEM-GBMs carried spontaneous missense p53 mutations, indicating a selective role in GBM progression. To determine whether tumors resembled any of the molecular subclasses of human GBM, transcriptome analyses were performed and revealed concordance with the highly aggressive human mesenchymal subclass. Because of their inducibility, high-penetrance, pathway specificity, and molecular and histological similarity to human high-grade astrocytomas, these models represent a unique resource for further mechanistic analyses and for preclinical studies, including the validation of potential drug targets and diagnostic and therapeutic development.

NCI-CPFP
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Tumor Biology and Metastasis

The DNA double-strand repair genes BARD1 and RAD51 as molecular targets for brain metastases from breast cancer

Brain metastases occur in 15-20% of metastatic breast cancer patients and up to one-third of those with triple-negative or HER2+ disease. Their prognosis is dismal with a one-year survival of merely 20%. Pharmacological agents against brain metastases do not exist currently, thus identifying brain metastasis-specific molecular targets has great translational significance. The DNA double-strand break (DSB) repair genes BARD1 and RAD51 were identified as part of a 13-gene signature predictive of rapid brain metastasis development (=3 years vs. >3 years after diagnosis, HR=3.03, p=0.0008) in primary tumors of HER2+ metastatic breast cancer patients. We found BARD1 and RAD51 mRNAs overexperessed (1.5 fold, p=0.0008 and 1.5 fold, p=0.001, respectively) in resected brain metastases compared to primary breast tumors from the same women and also significantly higher (1.5 fold, p=0.01 and 1.4 fold, p=0.008, respectively) in brain metastases compared to systemic (bone/lung) metastases. These observations led us to hypothesize that BARD1 and RAD51 are drivers of brain metastasis development in breast cancer patients. To functionally evaluate whether BARD1 and RAD51 promote brain metastases, the genes were overexpressed at physiologically relevant levels in a brain-metastatic variant of triple-negative MDA-MB-231 breast carcinoma cells. In vitro, this resulted in a ~30% increase in clonogenic colony formation (BARD1 p=0.001, RAD51 p=0.004), without affecting cell proliferation. Overexpression also conferred chemoresistance to DNA DSB inducing agents such as carboplatin (BARD1 p=0.005, RAD51 p=0.03) and doxorubicin (BARD1 p=0.03, RAD51 p=0.02), but not to agents that do not cause DNA DSBs, such as paclitaxel. In vivo, overexpression of BARD1 and RAD51 increased formation of large brain metastases, equivalent to clinically detectable lesions, (3.7 fold, p=0.002 and 4.0 fold, p=0.01, respectively) and micro-metastases (3.7 fold, p=0.001 and 3.1 fold, p=0.01, respectively) compared to vector controls in a mouse xenograft model. Importantly, this brain-metastasis promoting effect occurred without exogenous DSB induction, to which DSB repair genes would confer a growth advantage. Together these data suggest a driving role for BARD1 and RAD51 in the development of brain metastases from breast cancer. These genes are overexpressed in a brain metastasis-specific manner, and thus potentially present novel targets for brain metastasis treatment and prevention.

NCI-CCR
Chaudhary, Amit
Visiting Fellow
Vascular Disease and Biology

Blocking TEM8 Function Results in Broad Anti-Tumor Activity through Inhibition of Host Tumor Vasculature

Anti-angiogenic agents (AA) have become an important adjunct to anti-cancer therapy, but current FDA approved AA agents are unable to separate physiological and pathological angiogenesis and are limited by drug resistance and toxicities. Thus, the identification of new therapeutic targets that are more selective for pathological angiogenesis is needed. Tumor Endothelial Marker 8 (TEM8) is a highly conserved cell surface protein that was initially identified as a gene overexpressed in the vasculature of human tumors but was not detected in the angiogenic vessels of adult ovaries. To assess the functional role of TEM8, we disrupted the TEM8 gene in mice by targeted homologous recombination. TEM8 knockout (KO) mice were viable and reached adulthood without defects in physiological angiogenesis. Immunodeficient TEM8 KO mice showed impaired growth of human tumor xenografts of diverse origin including melanoma, breast, colon, and lung cancer. The microvascular density of tumors in TEM8 KO mice was significantly lower than that of wild type mice. These studies suggest that host-derived TEM8 promotes pathological but not physiological angiogenesis. Such specificity makes TEM8 an appealing target for the development of novel anti-angiogenic agents. To target TEM8, we used antibody phage display to develop fully human anti-TEM8 antibodies that bound the native extracellular domain of

mouse and human TEM8. In preclinical tumor challenge studies, these antibodies blocked the growth of various human tumor xenografts by inhibiting tumor induced angiogenesis. Anti-TEM8 antibodies inhibited tumor growth in TEM8 wild type (WT) mice, but had no effect on the slow growing tumors of TEM8 KO mice, demonstrating that these antibodies are function blocking and specifically target TEM8 in vivo. Anti-TEM8 antibodies also augmented the activity of other anti-angiogenic agents, vascular targeting agents, and conventional chemotherapeutic agents (5-Flourouracil and Irinotecan). In subacute toxicity studies these antibodies displayed no toxicity. Fluorophore-tagged Anti-TEM8 antibody selectively reacted with the tumor vessels in TEM8 WT but not TEM8 KO mice suggesting that they can also be used as a cancer imaging tool. Thus, anti-TEM8 antibodies provide a potential new adjunct for blocking pathological angiogenesis, with implications for both cancer diagnosis and therapy.

NHLBI
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Postdoctoral Fellow
Vascular Disease and Biology
Novel disease caused by mutations in CD73 leads to vascular calcification in adults
Vascular calcification is often presented as a secondary complication to diseases such as diabetes and chronic kidney disease; it contributes to morbidity, and is a predictor of premature death. We have identified a novel, monogenetic disease in which otherwise healthy individuals form arterial calcifications in their lower-extremity arteries. We performed genetic analysis on affected individuals from three separate families and identified three novel biallelic mutations in the NT5E gene in affected individuals. NT5E encodes for CD73, which converts extracellular AMP to adenosine. All identified mutations result in nonfunctional CD73. Extracellular adenosine acts as a signaling molecule by binding to one of the four adenosine receptors expressed on a wide range of cells; these patients are assumed to have reduced adenosine signaling, as CD73 is the major enzyme that metabolizes AMP to adenosine in the extracellular milieu. This is the first study that links extracellular adenosine signaling to pathological vascular calcification. Cultured fibroblasts from CD73-deficient individuals exhibited markedly reduced expression of NT5E mRNA, CD73 protein, and CD73 enzyme activity. Alkaline phosphatase is an enzyme known to be important in bone formation and calcification; cultured fibroblasts from CD73-deficient patients displayed an increase in alkaline phosphatase activity compared to control cells. We developed an in vitro calcification assay using patient specific CD73-deficient and control cells and show that CD73-deficient cells accumulate calcium phosphate crystals. Genetic rescue with a lentivirus overexpressing wild type CD73 normalized alkaline phosphatase activity in affected patient cells and prevented calcification. Exogenous adenosine treatment reduced alkaline phosphatase levels and more importantly, reduced in vitro calcification, indicating the role of adenosine signaling in the etiology of this disease. Recent studies suggest calcification in the vasculature mimics bone formation, however it is unclear as to the precise mechanisms involved; we have identified CD73, and thus adenosine signaling, as a potential regulator of this pathology. Loss of CD73 function due to biallelic NT5E mutations represents a new autosomal recessive disorder of vascular calcification, revealing the critical role of adenosine in inhibiting ectopic calcification within specific vessels.

NHLBI
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Vascular Disease and Biology
MicroRNA-223 is a Cholesterol Sensitive Master Regulator of Lipoproteins and Cholesterol Metabolism.
The etiology of progressive atherosclerosis can be traced to hypercholesterolemia. Cholesterol uptake, biosynthesis, and secretion are all integral parts of cholesterol metabolism and control systemic

cholesterol levels. Here we present a novel metabolic gene regulatory module controlling and linking all phases of lipoprotein and cholesterol metabolism. Cellular miR-223 expression is sensitive to intracellular cholesterol levels, and repressed miR-223 levels under cholesterol depletion (80% loss, P<0.001) are restored upon cholesterol delivery. Nuclear factor-kappaB (NF-kB) activity is significantly increased with cholesterol starvation and miR-223 harbors a putative NF-kB transcription factor binding site within its promoter. Inhibition of NF-kB activity significantly increased miR-223 levels (3.5-fold, P=0.01); therefore, NF-kB is a cholesterol sensitive negative regulator of miR-223 expression. miR-223 inhibits cholesterol biosynthesis (70% loss, P=0.01, radio-label assay) through direct targeting of phosphomevalonate kinase and sterol-C4-methyl oxidase-like, as determined by miR-223 overexpression studies quantified by real-time PCR (P=0.0009) and microarrays (P<0.0001). High-density lipoprotein is the key mediator of reverse cholesterol transport and hepatic cholesterol delivery, and its receptor, scavenger receptor BI (SR-BI), is a direct target of miR-223. SR-BI mRNA (70% loss), protein (45% loss), and functional activity (40% loss) are all significantly (P<0.005) reduced with miR-223 overexpression. Furthermore, miR-223 indirectly controls the expression of low-density lipoprotein receptor, ATP-binding cassette transporter, lecithin-cholesterol acyltransferase, and apolipoprotein AI through the direct targeting of Sp3 transcription factor. Overexpression and loss-of-function (siRNA & inhibitor) studies confirm Sp3's role in cholesterol-associated gene regulation. Inhibition of endogenous miR-223 activity and gene reporter (luciferase) assays confirm that SR-BI and Sp3 are direct targets of miR-223. In addition, miR-223(-/-)-null mice were determined to have elevated plasma cholesterol levels and altered lipoprotein profiles. In summary, we have identified a novel mechanism of cholesterol modulation mediated through miR-223's direct and indirect post-transcriptional regulation. These observations hold enormous potential for innovative drug targets, therapeutic strategies, and prevention of cardiovascular disease.

NCI-CCR
Abend, Johanna
Postdoctoral Fellow
Virology - DNA
Regulation of IRAK1 expression by KSHV microRNA decreases IL-1alpha-induced NF-kappaB activation and cytokine expression
Kaposi's sarcoma-associated herpesvirus (KSHV, HHV-8) is the causative agent of Kaposi's sarcoma (KS), the second most common AIDS-associated malignancy. KSHV expresses at least 18 different mature microRNAs (miRNAs) during latency. Analysis of a previously reported series of microarrays examining changes in cellular gene expression in the presence of KSHV miRNAs identified interleukin-1 receptor-associated kinase 1 (IRAK1) as a potential target. In luciferase assays with reporter plasmids containing the 3'UTR of IRAK1, miR-K12-1, -3, -3*, -6-3, and -9 significantly downregulated luciferase activity by 20 to 40%. Mutation of a predicted binding site for miR-K12-9 (miR-K9) in the 3'UTR of IRAK1 resulted in restoration of luciferase activity in the presence of miR-K9. These findings confirm direct binding of miR-K9 to the 3'UTR of IRAK1. In addition, IRAK1 endogenous protein expression was downregulated in cells transfected with miR-K9 as well as during de novo KSHV infection. These results validate IRAK1 as a newly discovered cellular target of KSHV miRNAs. IRAK1 is a key component of the IL-1R and TLR signaling cascades. We hypothesized that KSHV miRNA-mediated downregulation of IRAK1 would inhibit signaling through both of these pathways, resulting in lower levels of NF-kappaB activation and a subsequent decrease in specific cytokine production (for example, IL-1alpha-stimulated IL-6 and IL-8). In a KS tumor-derived endothelial cell line, downregulation of IRAK1 by miR-K9 resulted in lower levels of NF-kappaB activity upon stimulation with IL-1alpha. Results from RT-qPCR analysis showed a corresponding decrease in IL-6 and IL-8 mRNA levels upon IL-1alpha stimulation in the presence of miR-K9 or siRNA targeting IRAK1. TLR7/8 are innate immune receptors involved in recognition of viruses, and

thus miR-K9-mediated downregulation of IRAK1 may function as an immune evasion strategy for KSHV. Lower levels of NF-kappaB activity were observed in a human B cell line transfected with miR-K9 upon stimulation with a TLR7/8 agonist. This is the first evidence of viral miRNA-mediated suppression of TLR signaling. Future studies will address the role of miR-K9-mediated regulation of IRAK1 in KSHV reactivation from latently infected cells triggered by TLR7/8 stimulation. Due to its involvement in multiple immune signaling cascades, targeting of IRAK1 by KSHV miRNAs will likely have significant implications in KSHV pathogenesis.

NIAID
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Virology - DNA
A novel approach for the rapid mutagenesis and directed evolution of the structural genes of West Nile virus
Molecular clone technology has proven to be an extremely powerful tool for investigating the lifecycle of flaviviruses, their interactions with the host, and during vaccine development. However, the feasibility of employing existing strategies in large-scale mutagenesis efforts is limited by the technical challenges of manipulating the large and unstable molecular clone plasmids that encode these viruses. In this study, we have developed a novel strategy that streamlines the production of infectious West Nile virus (WNV), enabling the introduction of mutations into the viral structural genes at an unprecedented scale. An innovative aspect of this approach is that it does not involve traditional cloning steps that are both time-consuming and require the propagation of potentially unstable constructs in bacteria. To demonstrate the feasibility of using this technology to create large numbers of variants, we have constructed a library of variants encoding a single defined amino substitution at the 92 residues of the pr portion of the pre-membrane protein (prM) using this approach. We have also employed this approach to produce populations of variants with random genetic changes followed by selection of viruses with desirable properties. In this regard, we were able to rapidly identify mutations that allow escape from neutralizing antibodies. Populations of WNV encoding random changes in the E protein were produced in the presence of the potently neutralizing DIII-specific monoclonal antibody E16. Viruses resistant to neutralization were identified in a single passage. Together, we have developed a simple and rapid approach to produce infectious WNV. This system will enable new genetic approaches to study the structure, function, and immunogenicity of the structural proteins of flaviviruses.

NIAID
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Research Fellow
Virology - DNA
Varicella-zoster virus activates extracellular signal-regulated kinase (ERK)1/2 through the virus-encoding ORF12 protein
Varicella-zoster virus (VZV) is a neurotropic human alphaherpesvirus. Primary infection causes chickenpox, or varicella, and VZV reactivation results in shingles, or herpes zoster. During productive infection, VZV activates the mitogen-activated protein kinase (MAPK) pathway, and inhibitors to ERK1/2, p38, or JNK MAPK activity restrict viral replication. To identify viral proteins responsible for the activation of MAPKs, we screened a VZV expression library for the ability to activate an AP-1_luciferase reporter. We found that open reading frame 12 (ORF12) of VZV was able to enhance AP-1 reporter activity, and its effects on the activation of AP-1 reporter was strongly reduced by addition of inhibitors to MEK1/2, and, to a lesser extent, p38, but not by inhibitor to JNK. Consistent with the inhibitor results, transient expression of ORF12 resulted in phosphorylation of ERK1/2 and p38, but not JNK. Deletion of

ORF12 from the VZV genome resulted in smaller plaque size, impaired virus replication, and reduced levels of p-ERK1/2 compared to wild-type VZV in infected cells. In order to confirm that the mutant phenotype was due to the loss of ORF12, we inserted an epitope-tagged ORF12 in an ectopic site in the ORF12 mutant genome. The Insertion of the tagged ORF12 rescued the defects of the orf12 mutant in the phosphorylation of ERK1/2, plaque size, and viral replication. Western blot and immunofluorescence to the tagged ORF12 protein demonstrated that ORF12 was expressed efficiently and localized to the cytoplasm. In agreement with previous reports, inhibition of ERK1/2 activation with ERK1/2-specific inhibitor significantly reduced wild-type viral replication. This inhibition in viral replication was much reduced on orf12 mutant replication, furthermore, the orf12 mutant replicated to the similar levels to wild-type virus in the presence of ERK1/2 inhibitor. In conclusion, we have shown that VZV ORF12 is able to trigger the phosphorylation of ERK1/2 both in transfected or VZV-infected cells. The loss of ERK1/2 activation due to loss of ORF12 resulted in reduced viral replication, similar to that observed when wild-type viral infections were treated with ERK1/2 inhibitor. These data argues that ORF12 activation of MAPK is needed for optimal VZV replication.

NCI-CCR
Schowalter, Rachel
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Virology - DNA
Heparan Sulfate is an Attachment Receptor for Merkel Cell Polyomavirus
Strong evidence suggests that Merkel cell polyomavirus (MCV) is a causative factor in the development of a large proportion of highly lethal cancers arising from epidermal Merkel cells. While Merkel cell carcinoma is rare, it appears that infection with MCV is common, and many healthy people chronically shed MCV virions from the surface of their skin. In an effort to better understand the factors controlling MCV tissue tropism, we sought to characterize the cellular receptors that mediate MCV attachment to cultured cells. Although several previously studied polyomavirus species have been shown to bind to cell surface sialic acid residues associated with glycolipids or glycoproteins, we found that sialylated glycans are not required for initial attachment of MCV virions to cultured human cell lines. Instead, the presence or absence of a different glycosaminoglycan (GAG) known as heparan sulfate (HS) appears to be a critical determinant of the initial cell attachment step of the infectious entry process. This conclusion was drawn from a large set of experiments involving soluble competitors of heparan sulfate, enzymatic removal of cell surface GAGs, inhibition of cellular sulfation, and cell lines deficient in various GAGs or HS modifications. The kinetics of MCV binding to GAGs was also investigated using a basement membrane extract-binding assay, which revealed the existence of a second putative GAG-binding site. Intriguingly, we also discovered that while cell lines deficient in sialylated glycans readily bind MCV capsids, the cells are highly resistant to MCV infection. This suggests that sialylated glycans play a post-attachment role in the infectious entry process. Importantly, results observed using MCV reporter vectors were confirmed using a novel system for infectious propagation of native MCV virions. Taken together, the findings suggest a model in which MCV infectious entry occurs via initial cell binding mediated by HS, followed by secondary interactions with a sialylated entry co-factor. The study should facilitate the development of inhibitors of MCV infection and help shed light on the infectious entry pathways and cellular tropism of the virus.

NIAID
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Virology - DNA

Genome-wide analysis of the 5' and 3' ends of vaccinia virus early mRNAs delineates regulatory sequences of annotated and anomalous transcripts

Poxviruses are large DNA viruses that can cause deadly diseases in humans and animals. Vaccinia virus (VACV) is the prototype member of the poxvirus family and is also widely used as a vector for vaccine development and gene expression. VACV has a 200 kbp DNA genome that encode a multisubunit RNA polymerase, stage-specific transcription factors, and enzymes that cap and polyadenylate mRNAs within the cytoplasm of infected animal cells. To extend our previous whole transcriptome analysis [Z. Yang et al. (2010) PNAS 107: 11513-11518], we adapted tag-based methods in conjunction with SOLiD and Illumina deep sequencing platforms to determine the precise 5' and 3' ends of VACV early mRNAs and map the putative transcription start sites (TSSs) and polyadenylation sites (PASs). Individual and clustered TSSs were found preceding 103 annotated early genes. In the majority of cases, a 15-nucleotide consensus core motif was present upstream of the gene. This motif, however, was also present at numerous other locations indicating that it was insufficient for transcription initiation. Further analysis revealed a 10-nucleotide AT-rich spacer following functional core motifs that may facilitate DNA unwinding. Additional putative TSSs occurred in anomalous locations that may expand the functional repertoire of the VACV genome. However, many of the anomalous TSSs lacked an upstream core motif, raising the possibility that they arose by a processing mechanism as has been proposed for eukaryotic systems. Discrete and clustered PASs occurred about 40 nucleotides after an UUUUUNU termination signal. However, a large number of PASs were not preceded by this motif, suggesting alternative polyadenylation mechanisms. Pyrimidine-rich sequences were found immediately upstream of both types of PASs, signifying an additional feature of VACV 3' end formation and polyadenylation. This comprehensive, genome-wide, high-resolution analysis of the 5' capped and 3' polyadenylated ends of VACV early mRNAs has greatly extended previous knowledge and revealed new features and unanticipated complexity of VACV transcription. The results will be broadly applicable to other poxviruses.

NIAID
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Virology - RNA and Retroviruses

HUMAN NOROVIRUS INFECTION IN CHIMPANZEES: TISSUE TROPISM, VIRAL EVOLUTION, AND VACCINE DEVELOPMENT

Noroviruses are global agents of acute gastroenteritis, but the development of control strategies has been hampered by the absence of two key tools in the study of viral pathogens: a cell culture system and a robust animal model. For example, several diverse norovirus strains within two major genogroups (GI and GII) cause most disease, but it has been difficult to determine whether vaccines should include one (monovalent) or more (multivalent) strains to achieve broad protection. Studies in chimpanzees have played a key role in the characterization of several fastidious hepatitis viruses, and we investigated the feasibility of such studies for the equally-fastidious noroviruses. Seronegative chimpanzees inoculated iv with the human norovirus strain Norwalk virus (NV) did not show clinical signs of gastroenteritis, but the onset and duration of virus shedding in stool and serum antibody responses were similar to that observed in humans. NV RNA was detected in intestinal and liver biopsies concurrent with the detection of viral shedding in stool and NV antigen expression was observed in cells of the small intestinal lamina propria. Two infected chimpanzees re-challenged 4, 10 or 24 months later with NV were resistant to re-infection, and the presence of NV-specific serum antibodies correlated with protection. We evaluated the immunogenicity and efficacy of virus-like particles (VLPs) derived from NV (GI) and MD145 (GII) noroviruses as vaccines. Chimpanzees vaccinated intramuscularly with GI VLPs were protected from NV infection when challenged 2 and 18 months after vaccination, whereas

chimpanzees that received GII VLPs-vaccine or a placebo were not. We showed that seroconversion to norovirus in chimpanzees may correlate with resistance to re-infection by the same virus, and confirm GI and GII to be two different serotypes as previously suggested. This study establishes the chimpanzee as a viable animal model for the study of norovirus replication and immunity, and shows for the first time that a multivalent vaccine might be needed to protect against NV disease. Microarray experiments are in progress to further define correlates of immunity at the host gene level. The availability of an animal model for the study of human noroviruses is an important advance in the development of strategies to reduce the disease burden of this ubiquitous pathogen.

NIAID
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Virology - RNA and Retroviruses
Treatment of Influenza Virus Infection with Viral Peptides Linked to Dendritic Cells
The Ligand Epitope Antigen Presentation System (LEAPS) is a novel technology platform for immune cell modulation that uses synthetic peptide immunogens. LEAPS constructs consist of a peptide epitope linked to the binding ligand of an immune cell such as an antigen presenting cell that induces the immune system to mount a cellular, humoral or mixed immune response as a means to treat, control or prevent disease. We applied LEAPS technology to develop a new immune based treatment for influenza virus infection that we tested in mice. Influenza-LEAPS peptides were synthesized by conjugating the antigen presenting cell binding ligand (derived from human ß2-microglobulin) with 15 amino acid long peptides derived from influenza virus NP, M or HA proteins. These LEAPS peptides were used to stimulate the maturation of murine bone marrow derived immature dendritic cells (DCs), characterized by high levels of maturation markers such as CD83, CD86 expression on the cell surface. LEAPS-pulsed DCs were administered intravenously (IV) to mice infected with 10^6 TCID50 of A/PR/8/34 (H1N1) virus 24 h post infection. A significant reduction (p=0.005) of virus titer in lungs was observed on day 3 post infection in mice that received LEAPS-pulsed DCs compared to mice that received non-pulsed DCs. Interestingly, while all mice that received non-pulsed DCs died within 4 days post infection, 50% of the mice that received LEAPS-pulsed DCs survived. Our results suggest that LEAPS-pulsed DCs reduce the level of viral replication and provide protection from lethal influenza infection. To determine the homing of LEAPS-pulsed DCs post administration, we labeled LEAPS-pulsed DCs with CFSE and monitored the cells in lungs, spleen and lymph nodes 8, 24 and 48 h later. LEAPS-pulsed DCs begin to accumulate in the lungs of infected animals by 8 h while only a few labeled cells were detected in the lungs of uninfected mice, suggesting that the microenvironment provides signals to recruit LEAPS-stimulated DCs to the site of infection in this model. When we investigated the optimal time to administer LEAPS-pulsed DCs, we did not observe a significant difference between treatment at 8 h or 24 h post infection in terms of pulmonary viral titers, weight loss and survival. In summary, LEAPS-stimulated DCs administered after influenza virus infection ameliorated the disease. This approach may be of benefit in the treatment of influenza virus infection and as an adjunct therapy to antiviral drug.

NCI-CCR
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Virology - RNA and Retroviruses
Determining the frequency and mechanisms of HIV-1 and HIV-2 RNA copackaging by single virion analysis
Retroviruses package two full-length copies of the viral RNA genome. Dual infection of a single cell by two different viruses can result in newly assembled particles that are heterozygotes (contain one copy

of both viral genomes). This copackaging can yield genetic recombinants of the two viruses and act as a source of viral diversity. We sought to determine the frequency of HIV-1 and HIV-2 RNA copackaging and to dissect the mechanisms that allow the copackaging of RNA generated from distinct human retroviruses. HIV-1 and HIV-2 are derived from the primate viruses SIVcpz and SIVsm, respectively, and thus share only limited sequence identity. Despite this, HIV-1 and HIV-2 Gag polyproteins can coassemble into the same particle and their genomes can undergo recombination, albeit at an extremely low frequency. These observations imply that HIV-1 and HIV-2 RNAs can be copackaged into the same particle. To examine HIV-1 and HIV-2 RNA copackaging, we used the single virion analyses assay recently developed in our lab, in which the RNA content of each particle is determined by direct visualization. To achieve this, we inserted sequences recognized by RNA-binding proteins, bacteriophage MS2 capsid protein (MS2) or E. Coli BglG protein (Bgl), into the pol region of the viral genomes and tagged some of the Gag polyproteins with a cerulean fluorescent protein (CeFP). We coexpressed viral genomes with fluorescent protein-tagged MS2 and Bgl proteins and examined the viral particles using fluorescent microscopy to identify the RNA content of each particle. We found that when HIV-1 and HIV-2 RNA are present in viral particles at similar ratios, ~10% of the viral particles encapsidate both HIV-1 and HIV-2 RNA. Furthermore, copackaging of HIV-1 and HIV-2 RNAs can be promoted by mutating the 6-nt dimer initiation site (DIS) to discourage RNA homodimerization or to encourage RNA heterodimerization. The influence of the DIS sequence on copackaging implies HIV-1 and HIV-2 RNA heterodimerization can occur prior to packaging. We examined whether the ability of these heterologous Gag to coassemble is important for the copackaging of HIV-1 and HIV-2 RNAs. Consistent with the literature, we found that HIV-2 Gag is unable to package HIV-1 RNA but HIV-1 Gag is able to package both HIV-1 and HIV-2 RNA. Furthermore, HIV-1 Gag alone can mediate HIV-1/HIV-2 RNA copackaging. Therefore, coassembly of HIV-1 and HIV-2 Gag is not required for copackaging of RNA from these two viruses.

NIAID
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Virology - RNA and Retroviruses
Aerosol transmission of the 2009 pandemic H1N1 influenza virus in ferrets
The epidemiological success of pandemic and epidemic Influenza A viruses relies on the ability to transmit easily via aerosol. The 2009 pandemic H1N1 (pH1N1) virus originated by reassortment of a North American triple reassortant virus (TRS) and a Eurasian swine influenza virus that contributed the NA and M gene segments. Both the TRS and Eurasian swine viruses caused sporadic infections in humans but failed to spread from person to person as the pH1N1 virus did. Aerosol transmission of influenza A requires release of influenza particles into the air at a concentration that is sufficient to infect a susceptible host; this aspect of transmission has not been addressed in previous studies. We used the pH1N1 and its precursor viruses in the ferret model to evaluate the contributions of the different viral gene segments on shedding of influenza virus particles in the air and on the transmissibility of the pH1N1 virus. Using cyclone-based air samplers, we assessed the concentration of influenza particles released by the infected ferrets. Transmission efficiency of a virus was determined by the percentage of naïve ferrets with influenza virus detected in nasal secretions or that seroconverted 13 days post exposure. We found that 100% of ferrets infected with recombinant pH1N1 virus exhaled influenza particles and transmission occurred to 4/4 (100%) naïve ferrets. When ferrets were infected with a recombinant reassortant virus in which the NA and M genes of pH1N1 were replaced with TRS NA and M genes, only 25% of ferrets released influenza particles into the air and 2/4 (50%) naïve ferrets became infected. Both parental viruses, TRS and Eurasian swine, transmitted via aerosols to 50% (2/4) naïve ferrets. All viruses replicated to equal levels in the upper respiratory tract of infected ferrets,

suggesting that factor(s) other than viral replication are important for releasing influenza particles and transmission. Our studies show that the Eurasian swine virus NA and M genes are essential for release of influenza particles into the air and transmissibility of the pH1N1 virus via aerosol. Through this study we have recognized that different viruses may be exhaled to varying degrees and have identified a constellation of genes that influence the transmissibility of the pH1N1 virus. We conclude that transmissibility of influenza viruses is a complex trait that is influenced by genetic determinants in the virus as well as the ability of the virus to be aerosolized.

NIAID
Safronetz, David
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Virology - RNA and Retroviruses
Identification, characterization and pathogenesis of a unique Lassa virus strain from Mali
Lassa virus (LASV) is an arenavirus which is maintained in the multimammate rat (Mastomys natalensis) and is the etiological agent of Lassa fever (LF). In humans, LF can present with a wide range of symptoms, from apparently asymptomatic to severe hemorrhagic fever disease with multi-organ failure. LF is most commonly diagnosed in the West African countries of Nigeria, Liberia, Guinea and Sierra Leone, where combined up to 500,000 cases are identified per annum. In 2009, a case of LF was diagnosed post-mortem in a man who was evacuated from Mali to London with a 10-day history of fever. Field studies conducted in Mali identified infected rodents in a region south of Bamako with a focal hot-spot around the village of Soromba, where the lethal LF infection was probably contracted. LASV was isolated from rodents collected in the area and characterized by immunological, biochemical and structural means. Full genomic sequence analysis of the isolate confirmed a unique LASV strain, designated Soromba-R, which is identical to a partial sequence obtained from the Mali-originated British LF case, and phylogenetically most closely related to LASV strain AV. The virulence of Soromba-R was assessed in two animal models of LF, inbred strain 13 guinea pigs and cynomolgus macaques, and, compared to isolates from Sierra Leona (Josiah) and Liberia (Z-132), found to be less pathogenic as suggested by delayed time to death and decreased mortality rates. In both models, Soromba-R had similar tissue tropism and viral burden as determined by viral titration and histological analysis of organs samples. In addition, in non-human primates, Soromba-R infection was nearly indistinguishable from Josiah and Z-132 isolates by means of hematological, serum biochemical and coagulation profiles, and differences in endogenous pyrogens including IL-6, IFN?, and IL-8 were not observed. However, Soromba-R infection resulted in increased neutrophil activating or potentiating proinflammatory cytokines or growth factors including TNF-a, MIP 1-a, MIP 1-ß, IL-1ß, and G-CSF, as well as IL-5. These results demonstrate an expanded region of LASV endemicity and suggest Soromba-R might be less pathogenic due to increased innate host responses post-infection. This research was supported by the Intramural research Program of the NIH.

NIAID
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Virology - RNA and Retroviruses
TRIM79, a novel interferon stimulated gene, restricts tick-borne encephalitis virus replication by degrading the viral RNA polymerase
Tick-borne encephalitis virus (TBEV), genus Flavivirus, causes encephalitides in humans resulting in high mortality and morbidity rates and is therefore classified as a BSL4 pathogen. The flavivirus NS5 protein is essential to virus replication, encoding the RNA polymerase, as well as the major antagonist of interferon (IFN) signaling. Despite potent inhibition of IFN-dependent signaling, IFN production by

infected cells is effective in suppressing flavivirus dissemination to neighboring cells. Hence, understanding functions of genes induced by IFN (IFN stimulated genes or ISGs) may reveal new approaches for treating flavivirus infections that would bypass the NS5-mediated block to antiviral gene induction. To this end, we used proteomic approaches to identify ISGs that directly target NS5. This led to the identification of tripartite motif 79 (TRIM79), a novel mouse ISG with unique antiviral properties. TRIM79 facilitated the lysosomal degradation of NS5, a phenotype confirmed during infection of cells stably expressing TRIM79. As a result of lysosome degradation, TBEV proteins normally associated with NS5 and crucial for replication, including NS2B and NS3, were also degraded, despite their inability to directly bind TRIM79. TRIM79 expression significantly inhibited TBEV replication in tissue culture. Interestingly, antiviral activity was virus-specific because replication of the mosquito-borne flavivirus West Nile virus (WNV) was unimpeded by TRIM79 expression, consistent with a lack of WNV NS5 binding to TRIM79. Lastly, the importance of this ISG for the host antiviral response to TBEV was evaluated by knock-down experiments in mouse macrophages. Importantly, despite the induction of hundreds of genes by IFN, the absence of TRIM79 rendered IFN treatment ineffective against TBEV infection, highlighting the importance of a single virus-specific ISG for establishing an antiviral cellular state. Moreover, the exquisite specificity demonstrated by TRIM79 to discriminate between highly conserved NS5 proteins from TBEV and WNV reveals a remarkable ability of the innate IFN response to distinguish between closely related flaviviruses. Understanding the precise mechanism of TRIM79 restriction may lead to new strategies for targeted therapeutic intervention following exposure to flaviviruses evolved to circumvent IFN responses.